THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXII AUGUST, 1941 No. 4

EXPERIMENTS ON THE CANCERIZATION OF CELLS *IN VITRO* BY MEANS OF ROUS SARCOMA AGENT.

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Received for publication February 28, 1941.

IN the following experiments, which have been the subject of a preliminary note (Halberstaedter and Doljanski, 1939), it has been attempted to transform normal cells into sarcoma cells *in vitro* by the action of the Rous sarcoma agent. The experiments were carried out on cultures of chick embryo myocardium and the sarcoma agent was derived from Rous sarcoma cell cultures which were previously exposed to cell-lethal doses of radium rays. Fragments of Rous sarcoma cultures, freed of living cells by irradiation, but still containing active agent, were brought into contact with a fragment of normal cell culture. The latter was cultivated together with the added irradiated sarcoma material and its morphological and physiological behaviour was examined at successive coverslip passages. In previous experiments the respective radio-sensitivity of sarcoma cells and sarcoma agent had been tested.

The use of irradiation as a method of obtaining cell-free sarcoma agent appears to be preferable to that of ultrafiltration because the method is simple ; it entails no loss of agent activity ; it permits of obtaining an undiluted agent at a concentration equal to that which prevailed in the tissue, and it renders the elimination of living cells absolutely certain.

METHODS.

Material and tissue culture technique.

Cultures of myocardium from 7-day-old chick embryos were used for cancerization. The Rous sarcoma cell strain employed was kindly placed at our disposal by Dr. A. Fischer (Copenhagen). Cultivation was carried out according to Carrel's standard coverslip method in mixtures of chicken plasma and diluted embryo extract. Passages were made every third day.

Irradiation.

For the irradiation of Rous sarcoma cultures we used a radium plaque of 1 cm.^{2} containing 5·96 mg. radium element, with a filter of 0·05 mm. Monel, and 0·05 mm. mica, with an addition of 0·1 mm. mica on which the culture was grown. With this filtration, 12 per cent. of the soft and 58 per cent. of the hard β-ray were available and the γ-rays were in almost their original intensity. The distance between the radium container and the culture was 4·5 mm. We have used this method of irradiation in many previous tissue culture experiments and have already described it in detail (Halberstaedter and Doljanski, 1937).

Inoculation of cultures.

Most of the inoculations were made in adult hens ; 2 to 3-months-old chicks were also occasionally used. An incision was made in the skin, a thin layer of the pectoral muscle gently lifted, and the culture was then placed in the pocket thus formed. One to four inoculations were made on each fowl.

The cultures of the Rous sarcoma strain which we used produced tumours on inoculation in almost 100 per cent. The tumours were generally demonstrable 7 to 10 days after inoculation, and death of the chickens took place in 4 to 5 weeks.

EXPERIMENTAL.

Determination of the Immediate Lethal Dose of Radium Rays for Rous Sarcoma Cells.

In earlier experiments we were able to show that by using 5·96 mg. radium it was possible to completely check growth of normal cell colonies cultivated *in vitro*. Cultures of fibroblasts (Halberstaedter and Doljanski, 1937) and leucocytes (Doljanski and Halberstaedter, 1937) irradiated 32–35 hours show no sign of proliferation or cell-migration when incubated. This effect has been described as " immediate lethal effect."

We started these experiments by determining the " immediate lethal dose " for Rous sarcoma cultures. Two-day-old sarcoma cultures were transferred into fresh medium and, immediately after planting, the radium plaque was fixed over the cultures. Irradiation was carried out in the refrigerator at 6° C., and the cultures were then placed in the incubator and examined after 24 and 48 hours.

Examination of cultures irradiated for 12 hours showed a scanty outgrowth of fibroblasts and migration of round cells from the tissue fragment ; in most cases the coagulum became slightly liquefied (Fig. 1). After exposure for 18 hours the migration of a few cells could still be observed, but there was no liquefaction. Out of six cultures exposed for 24 hours there were no cells in four (Fig. 2), and single cells only in two. From 24 cultures irradiated for 36 to 60 hours not a single cell migrated.. A dose corresponding to an exposure of 36 hours is therefore sufficient to suppress growth activity of Rous sarcoma *in vitro* completely and immediately.

Experiments on irradiation of tissue cultures have shown that cell colonies

exposed to doses not immediately lethal can proliferate over a certain period, but that after this time their proliferating ability is exhausted and they are incapable of further growth (Spear, 1935). The doses necessary to obtain this "delayed lethal effect" are far smaller than the immediate lethal dose. We are, therefore, justified in assuming that the immediate lethal dose established by us in the irradiation of Rous sarcoma cultures is sufficient to immediately inhibit all cell migration, and is also considerably larger than the dose necessary to destroy the power of multiplication of sarcoma cells. Unpublished experiments with rat benzpyrene sarcoma carried out in this laboratory have shown that the dose of Roentgen rays which prevents cell migration in explanted tumour fragments is at least 20 times greater than that which is lethal for the tumour, i. e. which prevents tumour take, the respective dose levels observed being 140,000 and 7000 r.

In order to make absolutely sure that all sarcoma cells capable of multiplying were eliminated in the irradiated cultures, a dose double that of the immediate lethal dose was used in our experiments.

Infectivity of Irradiated Sarcoma Cultures.

Two-day-old cultures of sarcoma cells were exposed to radium in the refrigerator and then immediately inoculated into chickens. Four cultures irradiated with an immediate cell lethal dose corresponding to an exposure of 36 hours all caused tumours on inoculation into fowls. The infectivity of such irradiated cultures depended entirely on the activity of the agent which remained present in the cultures and was not destroyed by the radium dose lethal for cells. The resistance of sarcoma agent to radium rays is considerable, for cultures exposed to irradiation for 72 hours and 96 hours caused tumours in two out of four and three out of three respectively.

It follows from these experiments that there exists a wide margin between the cell-destroying and agent-destroying dose. This permits a definite separation, by the use of a suitable radium dose, of active sarcoma agent from living cells. That there exists this difference in sensitivity to rays between active agent and sarcoma cells was shown by Rous (1913), Russ and Scott (1926), and Furth, Tuggle and Breedis (1938).

The Fate of Active Agent from Irradiated Rous Sarcoma Material in Cultures of Normal Tissue.

A 2-day-old Rous sarcoma culture was irradiated for 60 hours and transferred immediately after irradiation with the addition of a fragment of embryonic chicken heart culture. Not a single cell grew out of the irradiated Rous sarcoma fragment, whereas the added normal tissue proliferated actively; in a short time the new growth entirely surrounded the irradiated tissue. The cultures were transferred every 2nd or 3rd day for a period of 6–8 days. The resulting fibroblast colonies were perfectly regular and uniform in appearance.

After 6–8 days of cultivation (3rd passage) the infectivity of such cultures was tested by inoculation into fowls. The results, presented in Table I, show

TABLE I.—*Results of Inoculation of Normal Mesenchyme Cells Cultivated with Fragments of Sarcoma Cultures Previously Irradiated for 60 Hours. Each Culture was Transferred Three Times during the Stated Period of Growth.*

Number of irradiated culture.	Number of days of growth *in vitro*.	Result of inoculations into chickens.
7378	6	Positive.
7364	6	,,
7286	7	,,
7283	7	Negative.
7226	6	Positive.
7227	6	,,
7215	8	Negative.
7256	6	Positive.
7212	8	,,
7248	6	,,

that the addition of proliferating normal cells to irradiated sarcoma cultures containing active agent enables the active agent to persist for 6–8 days.

To prove that it is the actual presence of the normal cells which is responsible for maintaining the activity of the agent, Rous sarcoma cultures were irradiated for 60 hours and then incubated without the addition of normal cells. After varying periods of incubation the cultures were inoculated into chickens and the results, shown in Table II, demonstrate that cultures irradiated for 60 hours and then incubated for 12 hours still maintain their infectivity, whereas infectivity is completely destroyed after 48 hours' incubation.

TABLE II.—*Results of Inoculation of Rous Sarcoma Colonies Irradiated for 60 Hours and Kept Immediately after Irradiation for Various Lengths of Time at 37° C.*

Time of incubation in hours.	Number of inoculation experiments.	Number of positive results.
6	2	2
12	4	4
24	16	7
48	17	0

DESCRIPTION OF PLATES.

FIG. 1.—Experiment No. 14208. Rous sarcoma culture irradiated for 12 hours after 24 hours of incubation. × 39.

FIG. 2.—Experiment No. 14220. Rous sarcoma culture irradiated for 24 hours after 24 hours of incubation. × 35.

FIG. 3.—Experiment No. 11809. Rous sarcoma culture irradiated for 60 hours and subcultures with a piece of normal embryonic chicken heart for 4 passages. Fibroblasts originating from an added piece of embryonic heart. × 140.

FIG. 4.—Experiment No. 14537. Rous sarcoma culture irradiated for 60 hours and subcultured with a piece of normal embryonic chicken heart for 6 passages. (A) Round cells among fibroblasts. × 140. (B) Zone of round cells. × 380.

FIG. 5.—Experiment No. 11803. Rous sarcoma culture irradiated for 60 hours and subcultured with a piece of normal embryonic chicken heart for 4 passages. (A) Liquefaction area. × 73. (B) Cells bordering the liquefaction. × 500.

FIG. 6.—Experiment 11826. Rous sarcoma culture irradiated for 60 hours and subcultured with a piece of normal embryonic chicken heart for 5 passages. Resulting cultures with typical sarcoma appearance. × 16.

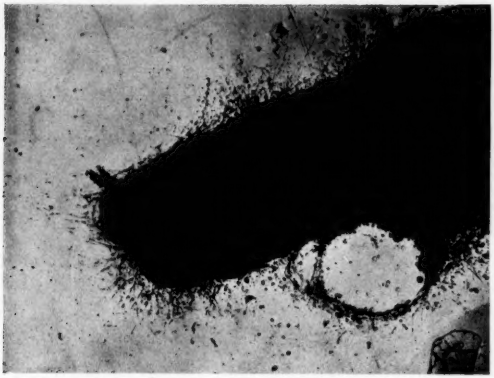

FIG. 1.

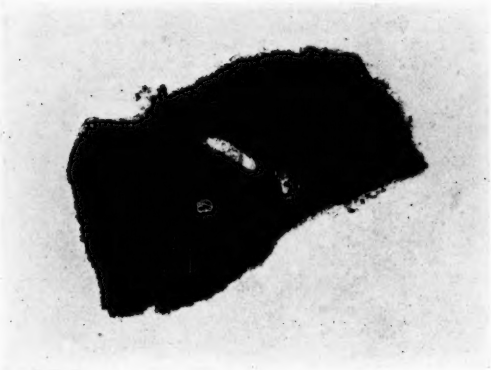

FIG. 2.

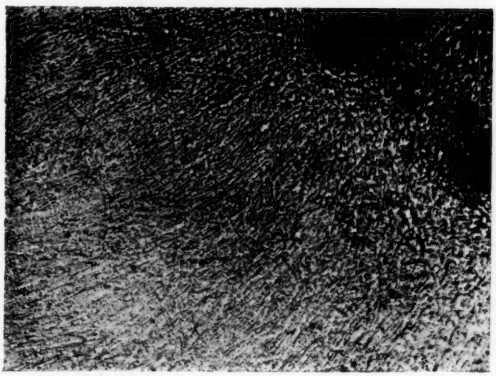

FIG. 3.

Halberstaedter, Doljanski and Tenenbaum.

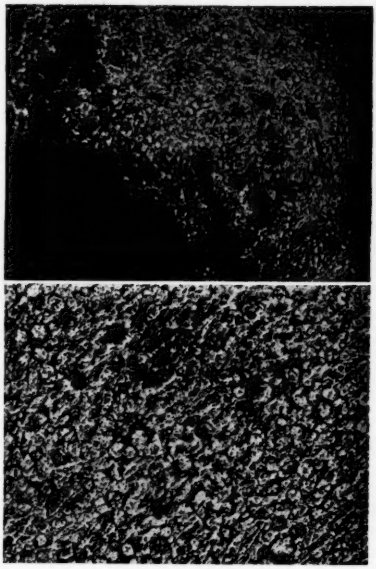

FIG. 4.

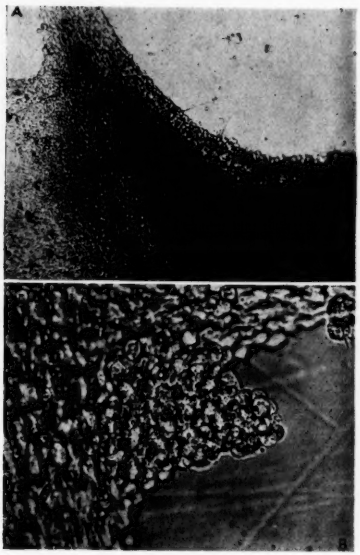

FIG. 5.

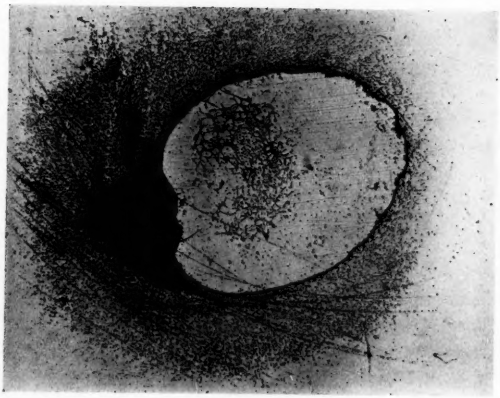

FIG. 6.

If living cells are not added therefore the Rous sarcoma agent in the irradiated cultures can only survive for a very limited time at 37° C. under our experimental conditions.

We have thus, by substituting proliferating normal cells for the killed sarcoma cells, succeeded in preventing the disappearance of active sarcoma agent in irradiated cultures.

The Transformation of Normal Cells into Sarcoma Cells under the Influence of Causative Agent of Rous Sarcoma in vitro.

We have shown that the causative agent of Rous sarcoma maintained its activity when brought in contact with proliferating normal cells, but the question still remains open whether normal cells "infected" with Rous sarcoma agent can themselves acquire the properties characteristic of Rous sarcoma cell colonies. To establish the malignant transformation of a cell culture occurring under the influence of sarcoma agent it is not enough to demonstrate that the culture is infective for the fowl. The pathogenicity of such a culture may be conditioned by the causative principle surviving or multiplying in the presence of normal cells. We would here be dealing with a "virus culture" analogous to cultures of non-oncogenic viruses, such as vaccinia, fowl plague, etc.

Malignant transformation of a cell *in vitro* could be inferred with certainty only if there were definite morphological and biological characteristics differentiating a malignant from a normal cell culture. Though the question of the existence of morphological peculiarities in malignant mammalian cells *in vitro* is still being fiercely debated, there is no doubt that the Rous sarcoma cell colony possesses extremely characteristic features. The cellular structure and the biological behaviour of a Rous sarcoma cell colony are completely different from those of a culture of any normal mesenchyme cells (Fischer, 1936).

The question whether normal cells are capable of acquiring the properties characteristic of Rous sarcoma cells under the influence of sarcoma agent can be answered by the study of the morphology and behaviour of normal cell colonies brought into contact with sarcoma agent and regularly transferred over a prolonged period. We therefore maintained through numerous passages cultures of normal myocardium brought into contact with Rous sarcoma cultures destroyed by 60 hours' irradiation but still containing active agent. The morphology and infectivity of such cultures were systematically investigated.

Our experiments have shown that the acquired pathogenicity is a permanent characteristic of such cell strains. Inoculations of 20th passage cultures (cultivated 60 days) results in tumours. Moreover, by continued cultivation of such culture strains *in vitro* it is possible to prove that in the course of cultivation the normal cell cultures acquire all the characteristics of true Rous sarcoma cell colonies.

The course of the cancerization process is as follows: In the first 3–5 passages the infected normal cells proliferate in the same manner as normal fibroblasts. The growth area of these cultures at first consists of radial and

" grass-like " proliferating spindle cells (Fig. 3). After 4–5 passages, however, this radial structure may be lost in certain areas of the colony. The growth zone, particularly that bordering on the original fragment, consists then not only of spindle cells but also of numerous thickly packed, round cells (Fig. 4). In the next passages characteristic liquefaction appears (Fig. 5). The liquefaction areas become progressively larger until they surround the entire culture. This transformation process results in a typical Rous sarcoma colony (Fig. 6) which can be maintained as such indefinitely. Such colonies were formed of highly polymorphic cells of characteristic structure and behaviour ; they liquefy the plasma clot with enhanced intensity and can be cultivated only if normal tissue is regularly added.

The following protocols illustrate the course of the cancerization process :

Experiment I (3.vii.1938).

1st day : A radium plate was placed on a Rous sarcoma culture (No. 7776).

4th day : After 60 hours of irradiation the radium was removed and the culture transferred into fresh medium with addition of a piece of embryonic chicken heart.

6th day : Culture transplanted.

8th day : Culture divided and both fragments transplanted with addition of pieces of embryonic chicken heart.

10th day : One culture divided and transplanted, the other simply transplanted.

12th day : Cultures transplanted.

15th day : Two of three cultures showed liquefaction. Cultures transplanted.

17th day : Following transplantation the residue of plasma coagulum with remaining cells of two liquefied cultures (Nos. 7858 and 7859) was inoculated into chicken No. 933 (result positive).

20th day : Cultures transplanted.

23rd day : All three cultures extensively liquefied. Culture No. 7879 was inoculated into chicken No. 936 (result positive).

25th to 46th days: Every third day the cultures were either simply transplanted or divided and transplanted with addition of embryonic chicken heart. All cultures maintained the ability to liquefy the plasma coagulum, and had the appearance of sarcoma cultures.

48th day : Cultures Nos. 8129 and 8130 (20th passage) were inoculated in chicken No. 953 (result positive).

Results.—A piece of fresh embryonic chicken heart was added to a Rous sarcoma culture which had been irradiated for 60 hours with radium and cultured for 20 passages. The cultures showed signs of liquefaction on the 11th day after irradiation. On further cultivation cultures assumed the appearance of typical Rous sarcoma colonies. Cultures which were inoculated on the 17th and 21st day of cultivation (7th–8th passage as well as two other cultures which were inoculated on the 48th day (20th passage) produced tumours.

Experiment II (4.viii.1938).

1st day : A radium plate was placed on a Rous sarcoma culture (No. 7957).

4th day : After 60 hours of irradiation the radium was removed and the culture transferred into fresh medium with addition of a piece of embryonic chicken heart.

6th and 8th days : Culture transplanted.

11th day : Culture divided and both fragments transplanted with addition of pieces of embryonic chicken heart.

14th day : One of the divided cultures divided once more and all three were transplanted.

16th day : Cultures transplanted. One showed signs of liquefaction.

19th day : Cultures transplanted.

22nd day : Cultures transplanted. A liquefied culture was divided.

24th day : The two subcultures resulting from the latter were transplanted.

26th day : Both cultures which had assumed the appearance of Rous sarcoma cultures were inoculated into chicken No. 956 (result positive).

Results.—A piece of fresh embryonic chicken heart was added to a Rous sarcoma culture

which had been irradiated for 60 hours with radium and cultures together for 9 passages. The resulting cultures showed signs of liquefaction on the 12th day after irradiation. Two cultures having the appearance of Rous sarcoma cultures were inoculated together on the 22nd day of cultivation (9th passage) and produced tumour.

Experiment III (8.viii.1938).

1st day : A radium plate was placed on a Rous sarcoma culture (No. 7993).

4th day : After 77 hours of irradiation the radium was removed and the culture transferred into fresh medium with addition of a piece of embryonic chicken heart.

7th day : Culture transplanted.

10th day : Culture transplanted.

12th day : Cultures divided and both fragments transferred into fresh medium with addition of a piece of embryonic chicken heart.

15th, 17th, 19th and 21st days : Cultures transplanted.

24th day : One of the cultures which showed extensive liquefaction was divided and transplanted. The second non-liquefied culture was simply transplanted.

26th day : The extensively liquefied subcultures Nos. 8227 and 8228 were both inoculated into chicken No. 958 (result positive) and the unliquefied culture No. 8229 was inoculated into chicken No. 957 (result negative).

Results.—A piece of fresh embryonic chicken heart was added to a Rous sarcoma culture which had been irradiated for 77 hours with radium, and cultured for 9 passages. The cultures showed signs of liquefaction on the 20th day after irradiation. The liquefied cultures were inoculated on the 22nd day of cultivation (9th passage) and produced a tumour.

DISCUSSION.

Carrel (1926) was the first to show that Rous sarcoma agent remains active *in vitro* when brought into contact with normal cells. Carrel added Rous tumour filtrates to flask cultures of normal tissues, and found that the causative agent which lost its activity after 24 hours at 37° was able to maintain its virulence in the presence of living cells. Fragments of such cultures as well as a small piece of wool impregnated with the nutrient medium inoculated into fowls produced tumours. If fresh cell material was added to the medium from time to time and the cultures were transferred into new flasks every 2nd or 3rd week, the filterable agent multiplied and remained active for a considerable time. Carrel (1924) also reported that when he added sarcoma powder to cultures of macrophages he was able to observe that in such cultures there occurred a sarcomatous transformation of the macrophages as well as a multiplication of sarcoma agent. In his detailed paper regarding the conditions of reproduction *in vitro* of the Rous sarcoma agent Carrel (1926) referred to his previously cited work, but emphasizes that it is not always possible to establish malignant transformation in infective cultures, and concluded that it is not certain that activity of Rous virus is always accompanied by cell lesions.

Ludford (1937) confirmed Carrel's finding that the active agent persists in colonies of normal cells cultivated over a long period. He came to the conclusion that *in vitro* there takes place an infection of normal cells by sarcoma agent, which means that the sarcoma agent combines with the cell. This relation is so intimate that even treatment with immune serum which is otherwise capable of inactivating any free sarcoma agent is unable to abolish the infectivity of such a culture. Although Ludford considered it as very

probable that such infected fibroblasts are equivalent to malignant cells, he believes that the transformation of normal into malignant cells has not been completely demonstrated by his experiments. He was unable to furnish absolute proof for this change because in his cultures characteristic signs of such a transformation were not perceptible.

The experiments reported by us furnish confirmation of the results of Carrel and Ludford as to the maintenance of the pathogenicity of active Rous sarcoma agent in cell cultures infected with the latter, and show further that cultures of normal cells which are combined with Rous sarcoma agent are transformed in the course of cultivation into Rous sarcoma cultures. By the use of the coverslip method of cultivation and extending the observation times over numerous passages, it was found that the normal cells undergo a transformation and acquire all the physiological and morphological properties of Rous sarcoma cells. In view of these results we consider it justified to describe the change induced in cell cultures by the Rous sarcoma agent as true cancerization.

The transformation of a normal cell culture into a colony of sarcoma cells is a process which can be followed step by step, and has, as a whole, been described by us. The detailed description of the changes undergone by the single cells is beyond the scope of the present investigation and will be dealt with separately in a further communication.

We may note in conclusion that our analysis of the relation between active agent of Rous sarcoma and normal cells in culture renders a new interpretation of the processes occurring in the cultivation of Rous sarcoma *in vitro* necessary. The usual method of cultivation of Rous chicken sarcoma is that of Fischer (1930), in which the cultures are kept permanently alive and virulent by the regular addition of normal chicken tissue to the proliferating sarcoma cell colonies. According to Fischer the function of the added normal tissue is a supporting and nutritive one. The sarcoma cells, which liquefy the plasma clot intensively, find the mechanical support necessary for their growth in the added tissue. The latter can also be digested and assimilated by the sarcoma cells. Herewith the function of the added material was believed to be exhausted. Our experiments necessitate reconsideration of this view. They show clearly that in sarcoma cultures to which normal cells are added a direct contact between active agent and normal tissue is established. The normal cells become combined with the agent and are gradually transformed into sarcoma cells. Therefore, in cultures of sarcoma maintained according to Fischer's method aside from the multiplication of the original sarcoma cells a continual cancerizing of the added normal cells takes place. It is not the continuity of sarcoma cells but the continuity of sarcoma agent in the presence of constantly changing cell material which is essential to cultivation of Rous sarcoma *in vitro*.

SUMMARY.

Cells of a Rous chicken sarcoma culture are destroyed by doses of radium which are without effect on the activity of the sarcoma agent. With the

method employed the "immediate lethal effect" for sarcoma cells was attained after 36 hours' irradiation; sarcoma agent was still active after 96 hours' irradiation.

The pathogenicity of 36 hours' irradiated cultures disappears when cultures are placed in the incubator for 48 hours immediately after irradiation. It is possible to maintain the activity of sarcoma agent by adding normal cells to irradiated sarcoma cultures.

Normal cell cultures combine *in vitro* with active sarcoma agent from irradiated cultures, and acquire in the course of passages all the morphological and biological characteristics of sarcoma cell colonies which are maintained *in vitro*. Such cell strains remain permanently pathogenic.

When Rous sarcoma is cultivated *in vitro* with the addition of normal tissue according to the method of Fischer, there occurs besides a multiplication of the original sarcoma cells, also a continuous cancerization of the normal tissue added.

The authors wish to express their appreciation of the technical assistance given by Miss B. Malachowski and Mrs. L. Sternberg.

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THE FORMATION OF PROTEIN COMPLEXES IN HEATED SOLUTIONS OF RABBIT SERUM PROTEINS.

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Received for publication June 20, 1941.

THE results of heating euglobulin fractions of rabbit antisera showed that the presence of other serum fractions during heating leads to the formation of changed antibodies which can unite with their antigens but not flocculate them (Kleczkowski, 1941). Heating euglobulin fractions of antisera in the absence of other serum fractions does not produce such changes in the serological behaviour of antibodies. In order to understand the possible interaction between antibodies and other serum proteins, the behaviour of normal rabbit serum proteins on heating has been studied, and this paper describes the differences obtained when rabbit serum euglobulin and albumin solutions are heated separately and together.

METHODS AND MATERIAL.

Preparation of protein fractions.—Normal rabbit serum was 1/3 saturated with ammonium sulphate, the precipitate filtered off and washed with 1/3 saturated ammonium sulphate solution. The precipitate was dissolved in water and dialysed, and the insoluble euglobulin was filtered off. The filtrate was used as soluble euglobulin.

The filtrate from serum 1/3 saturated with ammonium sulphate was 1/2 saturated and the precipitate of pseudoglobulin filtered off. The filtrate was then fully saturated with ammonium sulphate, the precipitate redissolved in water and dialysed. The solution was used as albumin fraction.

The nitrogen content of the dialysed solutions was determined by micro-Kjeldahl, and this was translated into protein content by multiplying by 6.25.

The protein solutions were heated in 0·9 per cent. NaCl solution at pH 6·8. They were heated in thin-walled tubes in a water-bath, and the time of heating was taken from the moment when the fluids reached the temperature of the bath, which was usually about one minute after immersion.

To determine the amount of protein precipitated from heated protein solutions by the addition of ammonium sulphate, the fluid was allowed to stand undisturbed for at least two days, when the precipitate had settled. Then to a measured volume of the supernatant fluid trichloracetic acid was added to make a concentration of 3·3 per cent., and the fluid was heated for a few minutes at 100° C. The resulting precipitate was centrifuged down and washed repeatedly

with 3·3 per cent. trichloracetic acid until no test for SO_4'' was obtained with $BaCl_2$. The amount of protein was then determined by micro-Kjeldahl.

RESULTS.

Table I shows that when solutions of euglobulin and albumin were heated for 10 minutes at 80° C. in identical conditions a precipitate separated from the first (Exps. Nos. 2 and 5) but not from the second (Exp. No. 1), and when mixtures of the two were heated their appearance depended on the proportions in which they were mixed (Exps. Nos. 3 and 4). All the heated solutions, however, gave a strong nitroprusside test (made as described by Harris, 1923), showing that they all had undergone changes producing active disulphide groups.

TABLE I.—*The Behaviour of Rabbit Serum Albumin and Euglobulin in Solutions Heated Separately and Together.*

The protein solutions were heated for 10 minutes at 80° C. in 0·9 per cent. NaCl at pH 6·8.

No. of experiment.	Amount (mg.) of protein in 10 ml. of solution.		Appearance of the fluid after heating.	Amount (mg.) of protein in the precipitate from 10 ml. of the solution.
	Albumin.	Euglobulin.		
1	50·0	0	Very slight opalescence	0
2	0	50·0	Precipitation	39·0
3	25·0	25·0	,,	34·0
4	37·5	12·5	Opalescence*	0
5	0	12·5	Precipitation	8·6

* 5·0 mg. of a yellow gelatinous sediment was obtained by centrifugation at 8000 r.p.m.

When the ratio albumin/euglobulin in the mixture was sufficiently in favour of albumin, for instance when it was 3/1 (Exp. No. 4), no precipitate separated on standing. However, by centrifugation at 8000 r.p.m. a yellow gelatinous sediment was obtained. This could be taken up in water to form a stable, slightly opalescent suspension. The mixture of equal parts of the two fractions (Exp. No. 3) gave a precipitate larger than the total amount of either constituent. Therefore, although albumin heated alone gave no precipitate, it is evident that protein separating as a precipitate from the heated mixture originated from both constituents. This suggests that when the two fractions are heated together a complex composed of heat-changed protein originating from both fractions is formed and its stability in suspension depends on the ratio of the two constituents. The experiments described below, in which precipitability by ammonium sulphate was used as an indication of changes produced by heating, show that this complex is produced *during* heating, for albumin and globulin solutions mixed after being heated separately do not influence each other's properties.

For estimating the precipitability by ammonium sulphate, 4 ml. samples of 0·5 per cent. protein solutions were heated, diluted to 10 ml. with water, and saturated ammonium sulphate solution was added to the required concentration. The amounts of precipitate are shown in Table II.

TABLE II.—*Precipitability of Euglobulin, Albumin, and of a Mixture of Equal Parts of the Two, after Heating for Varying Lengths of Time at 75° C.*

The proteins were heated as 0·5 per cent. solutions in 0·9 per cent. NaCl at pH 6·8.

Protein.	Precipitate formed.	The amounts of precipitate (as percentage of total protein) after heating. Minutes heating.				
		30.	40.	120.	160.	320.
Euglobulin	Spontaneously	0	22	55	55	55
Albumin-euglobulin	Spontaneously	0	0	0	0	0
Albumin	Spontaneously	0	0	0	0	0
Euglobulin	With 20 per cent. saturated $(NH_4)_2SO_4$	75	79	82	82	84
Albumin-euglobulin	With 20 per cent. saturated $(NH_4)_2SO_4$	0	0	0	0	43
Albumin	With 20 per cent. saturated $(NH_4)_2SO_4$	0	0	0	0	0
Albumin-euglobulin	With 28 per cent. saturated $(NH_4)_2SO_4$	56	65	76	76	80
Albumin	With 28 per cent. saturated $(NH_4)_2SO_4$	0	0	0	40	65
Albumin	With 42 per cent. saturated $(NH_4)_2SO_4$	64	65	67	67	69

When euglobulin solutions were heated at 75° C. for more than 30 minutes a precipitate separated spontaneously. No precipitate separated from albumin and albumin-euglobulin mixtures of equal parts heated for as long as 320 minutes, but the fluids became increasingly opalescent with increased heating and all heated solutions gave a positive nitroprusside test.

The precipitability of heated albumin-euglobulin mixture differed considerably from that of either albumin or euglobulin heated separately. After a given period of heating the degree of precipitability of the mixture was intermediate between those of euglobulin and albulmin. After 120 minutes' heating at 75° C. 20 per cent. saturated ammonium sulphate precipitated only euglobulin, and 28 per cent. saturation precipitated the mixture, but not albumin heated alone. The amount of protein precipitated from the mixture after 120 minutes' heating by 28 per cent. saturated ammonium sulphate was 76 per cent. of the total amount of protein. It is obvious, therefore, that the precipitate produced in the heated mixture must have been composed of material originating from both fractions, although this concentration of ammonium sulphate has no effect on solutions of albumin heated alone for 120 minutes. Thus the product of heating the mixture was formed at the expense of both constituents, and was more easily precipitable by $(NH_4)_2SO_4$ than albumin heated alone.

On the other hand, after 160 minutes' heating the albumin-euglobulin mixture is not precipitable by 20 per cent. saturated ammonium sulphate, whereas 55 per cent. of euglobulin heated alone precipitated spontaneously, and the amount of precipitate obtained by 20 per cent. saturated ammonium sulphate (together with the precipitate formed spontaneously) was 82 per cent. of total protein. Therefore, the product of heating the mixture forms a more stable suspension than euglobulin heated alone.

If albumin and euglobulin are heated separately and then mixed, they are precipitated by ammonium sulphate just as they are alone. This is illustrated by Table III. Table IIIA shows the results of heating for 20 minutes at 75° C. 28 per cent. saturated ammonium sulphate did not precipitate albumin heated alone (Exp. No. 1), but it precipitated heated albumin-euglobulin mixture (Exp. No. 2). 20 per cent. saturation did not precipitate the mixture (Exp. No. 3), but it precipitated euglobulin heated alone (Exp. No. 4), and the addition of albumin previously heated separately did not prevent the precipitation nor influence the amount of precipitate (compare Exps. Nos. 5 and 4).

TABLE III.—*Precipitation by Ammonium Sulphate of Heated Albumin, Euglobulin and a Mixture of the Two.*

Exp. No.	Volume (ml.) of heated 0·5 per cent. protein solution taken. Euglobulin.	Albumin.	Albumin-euglobulin mixture (1/1).	Volume of H_2O added (ml.).	Volume of satur. $(NH_4)_2SO_4$ added (ml.).	Per cent. of saturation with $(NH_4)_2SO_4$.	Appearance of the fluid after addition of $(NH_4)_2SO_4$.	Amount of protein in the precipitate (mg.).
The proteins were heated as 0·5 per cent. solutions in 0·9 per cent. NaCl at pH 6·8.								
			A. *Heating for* 20 *minutes at* 75° *C.*					
1	0	4	0	6	4·0	28	Clear	0
2	0	0	4	6	4·0	28	Precipitate	10·2
3	0	0	4	6	2·5	20	Slight opalescence	0
4	2	0	0	8	2·5	20	Precipitate	7·0
5	2	2	0	6	2·5	20	,,	6·8
			B. *Heating for* 15 *minutes at* 70° *C.*					
6	0	4	0	6	4·0	28	Clear	0
7	0	0	4	6	4·0	28	Slight opalescence	0
8	2	0	0	8	4·0	28	Precipitate	3·4
9	2	2	0	6	4·0	28	,,	3·6

Table IIIB shows the results of heating for 15 minutes at 70° C. 28 per cent. saturated ammonium sulphate did not precipitate albumin heated alone or the heated albumin-euglobulin mixture (Exps. Nos. 6 and 7), but it precipitated euglobulin heated alone, and the amount of precipitate was almost the same whether heated albumin solution was added or not (compare Exps. Nos. 8 and 9).

The results in Table IIIA and B show that only when the two proteins are heated together is their precipitability affected. The separation of floccules, or the production of a material forming stable suspensions which can be sedimented as a gel by centrifugation at moderate speeds, shows that large aggregates are produced during heating. The formation of a product composed of protein originating from both fractions, with properties different

from those of either constituent heated separately, indicates that combination between particles of both fractions occurs during heating. The appearance of active disulphide groups indicates at least an early stage of denaturation, so that the large colloidal complexes formed during heating are formed of at least partly denatured protein particles. The combination of protein particles to form these complexes thus coincides with a change of their original structure.

Recently Van der Scheer, Wyckoff and Clarke (1941) arrived at similar conclusions from their electrophoretic studies of heated horse serum, which showed that a new colloidal aggregation product resulting from denaturation of components of the serum is formed at the expense of both the albumin and the globulin.

SUMMARY.

The effects of heating normal rabbit serum albumin and euglobulin fractions separately and together are described. When a mixture of the two fractions is heated a product is formed with properties different from those of either fraction heated separately. This product is a complex formed by the two fractions uniting as they undergo denaturation.

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EFFECT OF HEAT ON FLOCCULATING ANTIBODIES OF RABBIT ANTISERA.

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Received for publication June 20, 1941.

EARLIER workers have found that antisera to different antigens vary in their resistance to heat. In general it has been found that bacterial somatic agglutinins lose their flocculating power with less heating than flagellar agglutinins. For example, Jones (1927) showed that flagellar agglutinin to hog-cholera bacillus still agglutinated after the antiserum was heated at 90° C. for 20 minutes, whereas somatic agglutinin did not after the same time at 75° C. It has been also shown that some antisera which have lost their ability to agglutinate after heating for several minutes at 70–80° C. still combine with bacteria to give the phenomenon of inhibition (Eisenberg and Volk, 1902 ; Jones, 1928). This ability to inhibit is destroyed at 90° C., i.e. approximately the temperature needed for destruction of flagellar agglutinins.

Bawden and Pirie (1938*b*) showed that plant viruses with rod-shaped particles form with their antisera fluffy, open floccules similar to those formed by the agglutination of bacterial flagellar antigens, whereas those with spherical (or almost spherical) particles form dense granular precipitates similar to those formed by the agglutination of bacterial somatic antigens. When antisera to these two types of viruses are heated, they behave in the same way as antisera to the two types of bacterial antigens. Strong antisera to rod-shaped tobacco mosaic virus flocculate until heated for 10 minutes at 90° C., whereas antisera to the spherical tomato bushy stunt virus do not flocculate after heating for 10 minutes at 75° C. This paper describes experiments made to investigate the causes underlying the differences in the apparent behaviour of different antisera on heating.

METHODS AND MATERIAL.

The antigens used were human serum globulin and albumin, a strain of pea nodule bacteria (*Rhizobium leguminosarum*) and purified preparations of the following plant viruses: tobacco mosaic, potato "X," tomato bushy stunt and tobacco necrosis.

The preparations of plant viruses were made by precipitation methods and kindly supplied by Mr. F. C. Bawden.

Human globulin was prepared by half saturating human serum with ammonium sulphate. The precipitate was dissolved in water and dialysed. NaCl was then added up to 0·9 per cent. to dissolve the precipitate formed during dialysis. Albumin was precipitated by full saturation with ammonium sulphate of the filtrate remaining after the precipitation of globulin. This precipitate was dissolved in water and dialysed, and NaCl to 0·9 per cent. added.

Suspensions of pea nodule bacteria were prepared by washing the cultures grown on agar slopes with 0·9 per cent. NaCl. The bacteria were then killed with 1 per cent. formaldehyde and washed with NaCl solution.

The antisera were prepared by injecting rabbits intravenously twice a week with solutions or suspensions of the antigens. From 4 to 6 injections in all were given and the rabbits were bled 8–10 days after the last.

Most of the antibodies in the antisera to all these antigens precipitated out with the euglobulin fraction. These fractions were precipitated with 1/3 saturated ammonium sulphate. The precipitate was filtered off, dissolved in 0·9 per cent. NaCl and dialysed against 0·9 per cent. NaCl solution until the test for SO_4'' with $BaCl_2$ was negative.

The fractions of normal rabbit serum used in this work and the methods of heating were the same as those described in the previous paper (Kleczkowski, 1941).

Flocculation tests were made by mixing 1 ml. of antigen solutions with either 1 ml. of antiserum solutions or 1 ml. of antiserum euglobulin solutions. The tubes were immediately placed in a water bath at 50° C. The appearance of floccules was taken as a positive result. The ability of heated solutions to combine with antigen without causing flocculation was tested by their ability to inhibit flocculation. The solutions to be tested were mixed with

antigen solutions and incubated for 3 hours at 50° C., when 0·1 ml. of a suitable dilution of unheated antiserum was added. The absence of flocculation was taken as evidence of inhibition.

RESULTS.

The effect of heating antisera to tobacco mosaic virus and tomato bushy stunt virus.

The results shown in Table I illustrate differences in the behaviour of tobacco mosaic virus and bushy stunt virus antisera after heating. The precipitating power of bushy stunt antiserum is completely destroyed by heating for 10 minutes at 75° C., but the ability to combine with antigen is preserved; this is shown by the inhibition of flocculation when unheated antiserum is subsequently added. The inhibition is specific; it is not caused by incubation with either heated normal serum or heated heterologous antisera. The inhibition is more pronounced after heating for 10 minutes at 80° C. (higher " inhibition titre ") and it still occurs after heating for 10 minutes at 85° C. It is destroyed by heating for 10 minutes at 90° C.

TABLE I.—*The Effect of Heating Antisera to Tobacco Mosaic and Bushy Stunt Viruses for 10 Minutes at Different Temperatures.*

The antisera were heated at a dilution 1/10 in physiological saline. The antigen solutions were used at 0·0025 per cent. for tobacco mosaic virus and 0·005 per cent. for bushy stunt virus. 1 ml. of antigen solution was added to a series of tubes each containing 1 ml. of antiserum at varying dilutions. + indicates precipitation and − no precipitation.

After 3 hours 0·1 ml. of control antiserum at a dilution 1/8 was added to the tubes where there was no precipitation. i indicates that there was still no precipitation (inhibition), and o that a precipitate was formed (no inhibition).

Temperature of heating.	Dilution of the antisera. Tobacco mosaic virus. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	Bushy stunt virus. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
Unheated	+	+	+	+	+	+	−	+	+	+	+	+	+	−
							o							o
75° C.	+	+	+	+	+	−	−	−	−	−	−	−	−	−
						o	o	i	i	i	i	o	o	o
80° C.	+	+	+	−	−	−	−	−	−	−	−	−	−	−
				o	o	o	o	i	i	i	i	i	o	o
85° C.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	o	o	o	o	o	o	o	i	i	o	o	o	o	o
90° C.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	o	o	o	o	o	o	o	o	o	o	o	o	o	o

By contrast, the precipitating power of tobacco mosaic virus antiserum is only slightly diminished by heating for 10 minutes at 75° C., and further diminished, but not destroyed, by heating at 80° C. Sera with higher titres are only inactivated after 10 minutes at 90° C. Thus there are two obvious differences in the behaviour of the two antisera. The bushy stunt antiserum

loses its ability to precipitate at a lower temperature than tobacco mosaic antiserum, but it then inhibits precipitation, whereas no such phenomenon can be observed with the heated tobacco mosaic antiserum. The amount of heat necessary to inactivate tobacco mosaic antiserum is apparently the same as that required to destroy the ability of bushy stunt virus antiserum to combine with antigen.

The influence of dilution of antisera in saline and in protein solutions on the effect of heating.

The results of experiments given in Tables II and III show that diluting antisera to tobacco mosaic and bushy stunt viruses in saline lessens the destructive effect of heating on their precipitating power; with bushy stunt virus antiserum it also prevents the appearance of the phenomenon of inhibition. On the other hand, diluting the antisera in protein solutions (normal rabbit serum, rabbit serum albumin and pseudoglobulin, human serum albumin) enhances the destructive effect of heating on the precipitating power and with bushy stunt antiserum it also enhances the appearance of inhibition phenomenon. The inhibition is stronger after diluting in albumin solutions than after diluting in whole rabbit serum or in pseudoglobulin solution.

TABLE II.—*The Effect of Heating Tobacco Mosaic Virus Antiserum Diluted in Physiological Saline and in Normal Rabbit Serum.*

The antiserum was heated for 10 minutes at 75° C. and 80° C. at varying dilutions in saline and in normal rabbit serum diluted 1/10 in saline.

Dilution.	Diluent.	Temperature of heating.	Dilution of antisera.				
			1/50.	1/100.	1/200.	1/400.	1/800.
—	Saline	Unheated.	+	+	+	+	—
							o
1/10	,,	75° C.	+	+	+	—	—
						o	o
1/50	,,	,,	+	+	+	+	—
							o
1/50	Serum	,,	+	+	—	—	—
					o	o	o
1/100	Saline	,,	..	+	+	+	—
							o
1/100	Serum	,,	..	+	—	—	—
					o	o	o
1/10	Saline	80° C.	—	—	—	—	—
			o	o	o	o	o
1/50	,,	,,	+	+	—	—	—
					o	o	o
1/50	Serum	,,	—	—	—	—	—
			o	o	o	o	o
1/100	Saline	,,	..	+	—	—	—
					o	o	o
1/100	Serum	,,	..	—	—	—	—
				o	o	o	o

Symbols as in Table I.

TABLE III.—*The Effect of Heating Bushy Stunt Virus Antiserum Diluted in Physiological Saline and in Protein Solutions.*

The antiserum was heated for 10 minutes at 75° and 80° C. at varying dilutions in saline, in normal rabbit serum diluted 1/10 in saline and in 0·6 per cent. solutions in saline of rabbit serum albumin, pseudoglobulin or human serum albumin.

Dilution.	Diluent.	Temperature of heating.	Dilution of antisera.				
			1/50.	1/100.	1/200.	1/400.	1/800.
..	Saline	Unheated	+	+	+	+	—
							o
1/10	,,	75° C.	—	—	—	—	—
			i	o	o	o	o
1/50	,,	,,	+	—	—	—	—
				o	o	o	o
1/50	Rabbit serum	,,	—	—	—	—	—
			i	o	o	o	o
1/100	Saline	,,	..	+	—	—	—
					o	o	o
1/100	Rabbit serum	,,	..	—	—	—	—
				i	o	o	o
1/10	Saline	80° C.	—	—	—	—	—
			i	i	i	o	o
1/100	,,	,,	..	—	—	—	—
				o	o	o	o
1/100	Rabbit serum	,,	..	—	—	—	—
				i	i	o	o
1/100	Rabbit albumin	,,	..	—	—	—	—
				i	i	i	o
1/100	Rabbit pseudoglobulin	,,	..	—	—	—	—
				i	i	o	o
1/100	Human albumin	,,	..	—	—	—	—
				i	i	i	o

Symbols as in Table I.

Streng (1909) and earlier workers showed that diluting bacterial antisera in saline slows down the heat inactivation of agglutinins and also prevents the appearance of the inhibition phenomenon. Diluting in physiological saline is one of the means of preventing coagulation, i.e. the formation of large complexes of protein particles, which follows denaturation. Other means of preventing coagulation, such as varying pH or addition of substances like urea, etc., also reduce the rate at which heat causes antisera to lose their flocculating power (Marrack, 1938). These facts suggest that loss of the flocculating power of antibodies, or the exchange of this power for the ability to inhibit after heating, is a result of production of large complexes of protein particles. The fact that the addition of unspecific proteins before heating has an effect similar to using more concentrated antiserum solutions suggests that the complexes containing antibody are produced by the union of different protein fractions. They are formed during heating, for the addition of unspecific proteins after heating has no such effect. The possibility of unspecific proteins affecting the behaviour of antisera on heating was indicated by Pick (1902) (quoted by Streng, 1909), who showed that typhus agglutinins "purified" by salting out (i.e. when a considerable amount of unspecific protein, like

albumin, had been removed) were much less affected by heating than the original antiserum.

The experiments described below were made to test these conclusions and to gain additional information about the role played by unspecific proteins during heating of antisera.

The effect of heating euglobulin fractions of antisera separately and in the presence of other fractions.

The rate of destruction of flocculating power by heat is much slower in the case of euglobulin fractions of antisera than with either whole antisera or mixtures of euglobulin fractions and albumin.

Tables IV and V illustrate this for tobacco mosaic virus antibodies. The difference can be seen when the solutions are heated for varying lengths of time at 70° C. (Table IV), but it is much more definite when they are heated at 75° C. (Table V). The precipitation titre of the whole antiserum and of its euglobulin fraction heated in the presence of albumin falls from 1/640 and 1/160 respectively to less than 1/10 within 20 to 40 minutes, whereas the titre of euglobulin heated alone is almost unaffected after 40 minutes and is only reduced from 1/160 to 1/40 after 160 minutes.

TABLE IV.—*The Effect of Heating at 70° C. for Varying Lengths of Time Tobacco Mosaic Virus Antiserum and Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

The antiserum was heated at a dilution 1/10 in saline. The euglobulin fraction of the antiserum (protein concentration 0·6 per cent.) was heated at a dilution of 1/10 in saline and in 0·4 per cent. rabbit albumin solution in saline.

Preparation of antiserum.	Time of heating (in minutes).	Dilution of the antiserum or of the euglobulin fraction.												
		1/80.	1/100.	1/120.	1/140.	1/160.	1/200.	1/240.	1/280.	1/320.	1/400.	1/480.	1/560.	1/640.	1/800.
Whole antiserum	0	+	..	..	..	+	..	..	..	+	..	..	..	+	—
	15	+	..	..	..	+	..	..	..	+	+	+	—	—	..
	30	+	..	..	..	+	..	..	..	+	+	—	—	—	..
	60	+	..	..	..	+	..	..	..	+	—	—	—	—	..
	120	+	..	..	..	+	+	+	+	—	..	..	..	—	..
	240	+	..	..	..	+	+	—	—	—	..	..	..	—	..
Euglobulin diluted in saline	0	+	+	+	+	—									
	15	+	+	+	+	—									
	30	+	+	+	—	—									
	60	+	+	+	—	—									
	120	+	+	+	—	—									
	240	+	+	—	—	—									
Euglobulin diluted in albumin.	0	+	+	+	+	—									
	15	+	+	+	—	—									
	30	+	+	—	—	—									
	60	+	+	—	—	—									
	120	+	—	—	—	—									
	240	+	—	—	—	—									

Symbols as in Table I. Only the results of precipitation are recorded. There was no inhibition.

TABLE V.—*The Effect of Heating at 75° C. for Varying Lengths of Time Tobacco Mosaic Virus Antiserum and the Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

The solutions used for heating were as described in Table IV.

Preparation of antiserum.	Time of heating (in minutes).	Dilution of the antiserum or of the euglobulin fraction. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.
Whole antiserum	0	+	+	+	+	+	+	+	− o
	10	+	+	+	+	+	+	− o	− o
	20	− i	+	+	+	− o	− o	− o	− o
	40	− i	− o	− o	− o	− o	− o	− o	− o
	80	− i	− o	− o	− o	− o	− o	− o	− o
	160	− o	− o	− o	− o	− o	− o	− o	− o
Euglobulin diluted in saline	0	+	+	+	+	+	− o		
	10	+	+	+	+	+	− o		
	20	+	+	+	+	±	− o		
	40	+	+	+	+	±	− o		
	80	+	+	+	+	− o	− o		
	160	+	+	+	− o	− o	− o		
Euglobulin diluted in albumin	0	+	+	+	+	+	− o		
	10	+	+	+	− o	− o	− o		
	20	− i	− o	− o	− o	− o	− o		
	40	− i	− i	− o	− o	− o	− o		
	80	− i	− i	− o	− o	− o	− o		
	160	− i	− i	− i	− o	− o	− o		

Symbols as in Table I.

Table V shows inhibition as a result of heating tobacco mosaic virus antiserum. The effect is slight compared with that of bushy stunt virus (compare Tables I and VI) and it is only transient. In the mixture of euglobulin with albumin, however, the production of inhibition is much more definite and lasting, although still less than with bushy stunt virus. In the mixture the ratio albumin/globulin is 7/1, i.e. more than three times greater than in antiserum.

The presence of protein fractions other than euglobulin in the heated

solutions affects bushy stunt virus antibodies much more than tobacco mosaic virus antibodies. Table VI shows that heating at 70° C. is here sufficient to demonstrate considerable differences. The precipitating power of the euglobulin fraction of bushy stunt virus antiserum heated alone is but little affected

TABLE VI.—*The Effect of Heating at 70° C. for Varying Lengths of Time Bushy Stunt Virus Antiserum and the Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

The antiserum was heated at a dilution 1/10 in saline. The euglobulin fraction of the antiserum (protein concentration 0·75 per cent.) was heated at a dilution 1/10 in saline and in 0·4 per cent. rabbit albumin solution in saline.

Time of heating (in minutes).	Dilution of whole antiserum.								Dilution of the euglobulin fraction heated alone.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
0	+	+	+	+	+	+	+	— o	+	+	+	+	+	+	— o
15	+	+	+	+	+	+	— o	— o	+	+	+	+	+	+	— o
30	+	+	+	+	+	— o	— o	— o	+	+	+	+	+	+	— o
60	+	+	+	— i	— o	— o	— o	— o	+	+	+	+	+	±	— o
120	+	+	— i	— i	— i	— o	— o	— o	+	+	+	+	+	±	— o
240	+	— i	— i	— i	— i	— o	— o	— o	+	+	+	+	+	±	— o

Time of heating (in minutes).	Dilution of the euglobulin fraction heated in presence of rabbit serum albumin.														
	1/10.	1/12·5.	1/15.	1/17·5.	1/20.	1/25.	1/30.	1/35.	1/40.	1/80.	1/160.	1/200.	1/240.	1/280.	1/320.	1/400.
0	+	..	..	..	+	..	..	..	+	+	+	..	..	..	+	— o
15	+	..	..	..	+	..	..	..	+	+	+	+	+	— o	— o	..
30	+	..	..	..	+	..	..	..	+	+	+	— o	— o	— o	— o	..
60	+	..	..	..	+	+	+	— i	— i	— i	— o	..	..	..	— o	..
120	+	+	+	— i	— i	..	..	..	— i	— i	— i	..	..	..	— o	..
240	+	— i	— i	— i	— i	..	..	..	— i	— i	— i	..	..	..	— o	..

Symbols as in Table I.

after 240 minutes, whereas the precipitation titre of the whole antiserum and of its euglobulin fraction heated in the presence of albumin falls from 1/640 and 1/320 respectively to 1/10. With the fall in the precipitation titre the phenomenon of inhibition appears. It first appears after about 60 minutes and its zone increases with the time of heating. It is definite before the precipitating power of heated solutions is totally destroyed. In such cases

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precipitation occurs in more concentrated antibody solutions and inhibition in the more dilute solutions.

When bushy stunt virus antiserum is heated at 75° C., its precipitating power is completely destroyed within 10 minutes and is replaced by inhibition (Table I), and the euglobulin fraction of the antiserum heated with 0·4 per cent. of albumin behaves similarly. But when the euglobulin fraction is heated alone, its precipitating power is almost unaffected after 10 minutes, and is not much decreased after 160 minutes (Table VII). However, a zone of inhibition appears after 10 minutes of heating ; this looks like an antibody excess zone, and it narrows as the time of heating increases.

TABLE VII.—*The Effect of Heating at 75° C. for Varying Lengths of Time the Euglobulin Fraction of Bushy Stunt Virus Antiserum.*

The euglobulin fraction (protein concentration 0·75 per cent.) was heated at a dilution 1/10 in saline.

Time of heating.	Dilution of the euglobulin fraction.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
0	+	+	+	+	+	+	— o
10	— i	— i	+	+	+	+	— o
20	— i	— i	+	+	+	±	— o
40	— i	+	+	+	+	— o	— o
80	— i	+	+	+	+	— o	— o
160	+	+	+	+	+	— o	— o

Symbols as in Table I.

Evidence has previously been given suggesting that normal rabbit serum proteins, while undergoing heat denaturation (in presence of 0·9 per cent. NaCl at pH near neutrality), combine with one another to form large mixed complexes (Kleczkowski, 1941). The results obtained with heated antisera suggest that antibodies can also unite with other proteins to form complexes. The serological behaviour of these complexes is determined by the other proteins present in the solution during heating and also by the type of antigen. When antibodies to either virus are heated with euglobulin fractions of the antisera only, they can still unite with and flocculate their antigens. That their properties are altered, however, is shown by slight changes in the precipitation. For example, tobacco mosaic virus precipitates are less nebulous and the floccules appear quicker than with unheated antibodies, and with bushy stunt virus antibodies the range of antibody excess inhibition is increased, so that precipitation is restricted to a narrower range of antigen/antibody ratios.

When antibodies are heated in the presence of protein fractions other than euglobulin much greater changes occur, and the differences between antigens of flagellar (tobacco mosaic) and somatic (bushy stunt) type become definite. The complexes formed in such mixtures of proteins can combine with their

antigens, but cannot cause flocculation, and their combination with antigen can prevent the latter from being precipitated subsequently by unchanged antibody. In a solution containing both changed and unchanged antibodies there is a competition between them, and the flocculation titre is the result of that competition. The titre, therefore, cannot be taken as a direct measure of the amount of antibody which remains unchanged, as authors who previously investigated the problem of the heat inactivation of antibodies have done. The result of the competition depends largely on properties of antigen particles. Consequently, after the same amount of heating in identical conditions tobacco mosaic virus antiserum can still precipitate its antigen, whereas bushy stunt virus antiserun has lost its precipitating power and inhibits. The results of the experiments described below show that such a competition does occur.

Effects of mixing heated and unheated antisera in varying proportions.

Antiserum to bushy stunt virus diluted 1/10 with saline was heated for 20 minutes at 80° C., so that its precipitating power was destroyed and inhibition was pronounced. The heated antiserum was then mixed with varying

TABLE VIII.—*The Effect of Mixing in Varying Proportions Heated and Unheated Bushy Stunt Virus Antiserum.*

The antiserum was heated for 20 minutes at 80° C. at a dilution of 1/10 in saline. This was mixed in varying proportions with unheated antiserum and the mixtures tested as described in Table I, or heated antiserum was first added to the solution of antigen and unheated antiserum followed after 1 hour.

Exp. number.	Ratio of heated antiserum to total amount of antiserum.	Dilution of the total antiserum. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
A. *Heated and Unheated Antiserum added Simultaneously.*								
1	0·0	+	+	+	+	+	+	—
								o
2	0·2	+	+	+	+	+	—	—
							o	o
3	0·4	+	+	+	—	—	—	—
					o	o	o	o
4	0·5	+	+	—	—	—	—	—
				i	i	o	o	o
5	0·6	+	—	—	—	—	—	—
			i	i	i	o	o	o
6	0·66	—	—	—	—	—	—	—
		i	i	i	i	i	o	o
7	1·0	—	—	—	—	—	—	—
		1	i	i	i	i	o	o
B. *Heated Antiserum Mixed first with Antigen Solution and Unheated Antiserum followed after 1 hour.*								
8	0·2	—	—	+	+	+	—	—
		i	i				o	o
9	0·4	—	—	—	—	—	—	—
		i	i	i	o	o	o	o

Symbols as in Table I.

amounts of unheated antiserum and the precipitating power of the mixtures was tested. The results are shown in Table VIII.

The competing action of changed antibodies is clearly shown. For example, the unchanged antiserum has a precipitation titre of 1/320, whereas a mixture of equal parts of heated and unheated antiserum has a titre of only 1/20, although there is sufficient unchanged antibody to give a titre of at least 1/160. Thus the competing action of the changed antibody has reduced the precipitation titre to one-eighth, showing the error made by taking the titre as a direct measure of the amount of unchanged antibody in the mixture.

Over the whole range of ratios in which heated and unheated antisera were mixed, there is a drop in titre due to the presence of changed antibody. This drop becomes greater with increasing amounts of heated antiserum in the mixture.

The results of heating bushy stunt virus antiserum for varying lengths of time at 70° C. agree closely with the results of mixing unheated and heated antiserum in varying proportions ; this can be seen by comparing Tables VI and VIII. With increasing time of heating at 70° C. (Table VI), as with increasing ratio of heated antiserum to the total amount of antiserum in the mixtures (Table VIII), the precipitation titre decreases. When the titre falls to about one-eighth of the original value the inhibition phenomenon appears, and the zone of inhibition widens with increasing time of heating or with increasing the ratio of heated to total antiserum. In both types of experiment inhibition occurs in the higher antiserum dilutions, while precipitation is still obtained in less dilute solutions. In less dilute solutions the concentration of unchanged antibody is still sufficient to cause precipitation, but in higher dilutions it is insufficient, and the competing action of changed antibody gives complete inhibition.

This similarity between the results shown in Tables VI and VIII suggests that during heating the ratio of changed to unchanged antibody increases, and that the fall in precipitation titre is an indication of this ratio.

Table VIII also shows that the addition of heated antiserum to antigen solution followed by unheated antiserum may give a result different from that when heated and unheated antisera are added simultaneously. Exps. Nos. 8 and 9 correspond to Nos. 2 and 3 respectively, except that in Nos. 8 and 9 unheated antiserum was added 1 hour after the heated antiserum, whereas in Nos. 2 and 3 heated and unheated antisera were added simultaneously. In some tubes the addition of the mixture gave precipitation, whereas there was inhibition when heated antiserum was added before the unheated. This difference again indicates competition between unchanged and changed antibodies. If enough changed antibody has already combined with antigen, unchanged antibody subsequently added cannot cause precipitation. However, if both kinds of antibody are added simultaneously, sufficient unchanged antibody may unite with antigen to give precipitation.

Similar experiments were made with tobacco mosaic virus antiserum and the results are shown in Table IX. Over the range of ratios of unheated to total antiserum from 0·0 to about 0·8 (Exps. Nos. 1–7) the precipitation titres are as they would be without any heated antiserum added, so that changed antibody present in heated antiserum has no visible influence on the precipitat-

TABLE IX.—*The Effect of Mixing in Varying Proportions Heated and Unheated Tobacco Mosaic Virus Antiserum.*

Methods as described for bushy stunt virus antiserum in Table VIII. Only the results of precipitation are recorded. There was no inhibition.

Exp. number.	Ratio of heated antiserum to total amount of antiserum.	Dilution of the total antiserum. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
A. *Heated and Unheated Antiserum Added Simultaneously.*								
1	0·0	+	+	+	+	+	+	−
2	0·2	+	+	+	+	+	+	−
3	0·4	+	+	+	+	+	−	−
4	0·5	+	+	+	+	+	−	−
5	0·6	+	+	+	+	+	−	−
6	0·7	+	+	+	+	−	−	−
7	0·8	+	+	+	−	−	−	−
8	0·9	−	−	−	−	−	−	−
B. *Heated Antiserum in the Exp. No. 8 Substituted by Equal Volume of Similarly Treated Normal Rabbit Serum.*								
9	0·9	+	+	−	−	−	−	−
C. *Heated Antiserum Mixed First with Antigen Solution and Unheated Antiserum followed after 1 Hour.*								
10	0·6	+	+	+	+	+	−	−
11	0·8	−	+	−	−	−	−	−

Methods of testing as in Table I.

ing action of unchanged antibody. However, when the ratio of heated to total antiserum is greater than 0·8 the effect of heat-changed antibody becomes apparent. In Exp. No. 8 there is no precipitation, although the amount of unchanged antibody is sufficient to give a titre of 1/20. This effect is due to the competing specific action of heated antiserum, for the presence of heated normal rabbit serum has no effect on precipitation (Exp. No. 9). Thus, unless heating is prolonged, for most purposes the titre of heated tobacco mosaic virus antiserum is a direct measure of the amount of unchanged antibody after heating.

Comparing Exps. Nos. 5 and 7 with Nos. 10 and 11 (Table IX) gives clearer evidence that heat-changed antibody can combine with tobacco mosaic virus without causing precipitation. The same amounts of heated and unheated antiserum were added to antigen in Exps. Nos. 5 and 10 and in Nos. 7 and 11, but in Nos. 5 and 7 the two were added as a mixture and in Nos. 10 and 11 the heated antiserum was added one hour before the unheated. As with bushy stunt virus, precipitation occurred in some tubes where heated and unheated antiserum were added at the same time, but not when heated antiserum was added first.

Inactivation of inhibiting power.

It has already been shown that heating for 10 minutes at 90° C. destroys

the inhibiting power of bushy stunt virus antiserum (Table I). Heating at lower temperatures for longer periods of time also does this. The inhibition titre at first increases, then remains approximately constant and later decreases. Thus at first there is a production of heat-changed antibodies, which have lost the precipitating power but still possess the ability to combine with antigen, while further heating produces a second change destroying all specific serological activity.

Behaviour of heated antibodies to other antigens.

To determine whether the results obtained with tobacco mosaic and bushy stunt virus antibodies apply at all generally, experiments were made with antibodies to potato virus " X," tobacco necrosis virus, pea nodule bacteria (*Rhizobium leguminosarum*) (317), human serum globulin and albumin.

Antisera to tobacco necrosis virus and potato virus " X " diluted 1/10 in saline were heated for 10 minutes at 80° C. Tobacco necrosis virus antiserum behaved like bushy stunt virus antiserum, the precipitation power being destroyed and replaced by inhibition. Potato virus " X " antiserum, on the other hand, behaved like tobacco mosaic virus antiserum ; its precipitation power was not destroyed, the only change being a decrease in the titre and an increased zone of antibody excess inhibition (cf. similar phenomenon with tobacco mosaic antiserum, Table V).

Tobacco necrosis virus, like bushy stunt virus, has quasi-spherical particles (Pirie *et al.*, 1938) and forms specific precipitate of the type " O," whereas potato virus " X," like tobacco mosaic virus, has anisodimensional particles (Bawden and Pirie, 1938*a*) and forms specific precipitates of the type " H." This indicates that the different results obtained with bushy stunt and tobacco mosaic virus antisera on heating are determined by their different shapes, and that the differences are probably generally applicable to all antigens of somatic (" O ") and flagellar (" H ") type. This is further supported by the fact that all the additional antigens tested give flocculation of the type " O," and their antisera on heating all behave very like bushy stunt virus antiserum.

Similar experiments were made with bacterial agglutinins. An antiserum to pea nodule bacteria diluted 1/10 in saline and also 0·1 per cent. solution of its euglobulin fraction in the presence and absence of other proteins were heated for 10 minutes at 80° C. The agglutinating power of the antiserum and of its euglobulin fraction heated in the presence of 0·4 per cent. rabbit albumin was destroyed and inhibition was definite, though less pronounced than with bushy stunt or tobacco necrosis viruses. On the other hand, the agglutination titre of euglobulin heated alone fell only from 1/3200 to 1/1600. The presence of 0·4 per cent. pseudoglobulin gave a rather different effect ; agglutination was destroyed, but there was no inhibition. When the euglobulin fraction of the antiserum and other fractions were mixed after being heated separately, agglutination occurred just as when the euglobulin was heated and tested separately.

Heating for 10 minutes at 90° C. destroyed both the ability to agglutinate and to inhibit.

TABLE X.—*The Effect of Heating at 70° C. for Varying Lengths of Time Antiserum to Pea Nodule Bacteria and the Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

Preparation of antiserum.	Time of heating (in minutes).	Dilution of the antiserum or of the euglobulin fraction.							
		1/50.	1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	1/6400.
Whole antiserum diluted 1/10 in saline	0	+	+	+	+	+	+	+	−
	15	+	+	+	+	+	+	−	−
	30	+	+	+	+	+	+	−	−
	60	+	+	+	+	+	−	−	−
	120	+	+	+	+	+	−	−	−
	240	+	+	+	+	−	−	−	−
0·1 per cent. euglobulin solution in saline	0	+	+	+	+	+	+	−	−
	15	+	+	+	+	+	+	−	−
	30	+	+	+	+	+	+	−	−
	60	+	+	+	+	+	+	−	−
	120	+	+	+	+	+	+	−	−
	240	+	+	+	+	+	+	−	−
0·1 per cent. euglobulin solution in 0·4 per cent. albumin	0	+	+	+	+	+	+	−	−
	15	+	+	+	+	+	−	−	−
	30	+	+	+	+	−	−	−	−
	60	+	+	+	+	−	−	−	−
	120	+	+	+	−	−	−	−	−
	240	+	+	+	−	−	−	−	−

Table X shows the effect of heating the same preparations of bacterial antibodies for varying lengths of time at 70° C. There was no change in the euglobulin fraction heated alone up to four hours, whereas the presence of albumin caused a large fall in the titre ; a similar fall occurred when the whole antiserum was heated.

Antisera to both human serum albumin and human serum globulin diluted 1/10 in saline and 0·1 per cent. solutions of euglobulin fractions of the antisera in the presence and absence of 0·5 per cent. rabbit serum albumin were also heated for 15 minutes at 75° C. The precipitating power of whole antisera was destroyed and the phenomenon of inhibition well pronounced. Heating the euglobulin fractions of the antisera alone did not destroy their precipitating power, but when rabbit serum albumin was added to the euglobulin solutions before heating, the precipitating power was destroyed and the inhibition phenomenon was produced. Heating the albumin and the euglobulin separately and then mixing them had no such effect.

DISCUSSION.

Solutions of flocculating (precipitating or agglutinating) antibodies of rabbit antisera twice undergo changes during heating. The first change corresponds with an early stage of denaturation of serum proteins, and coincides with the appearance of active disulphide groups and with the formation of large complexes of changed protein particles (Kleczkowski, 1941). The ability to combine with antigen is not destroyed by this change. The second change corresponds with a further stage of denaturation and coincides with the loss of this ability.

The serological behaviour of antibody after the first change depends on the proteins present in the solution when antibodies are undergoing heat denaturation, for combination between heat-changed antibody particles and those of other proteins seems to occur and the other proteins largely determine the behaviour. Evidence for such a combination between pneumococcal antibody and other proteins has recently been provided by electrophoretic studies on heated horse antisera (Van der Scheer *et al.*, 1941 ; Krejci *et al.*, 1941).

The name " euglobulin complex " will be used for the complex formed when euglobulin fractions of antisera are heated alone. When other fractions are present, either when whole antisera are heated or when other proteins are added to the euglobulin, " mixed complexes," i.e. composed of particles of different fractions, are formed.

The formation of " euglobulin complexes " only slightly affects the flocculating power of antibody particles. The titre of antibody solutions to both flagellar and somatic types of antigen is affected similarly, and as compared with unheated antibody there are only slight differences in zones of flocculation and type of floccules. It is the formation of " mixed complexes " that is responsible for the wide differences known to exist between the behaviour of heated antisera to somatic and flagellar types of antigen. The " mixed complexes " can still combine with antigens but are unable to cause flocculation. This is true both for flagellar and somatic type of antigens. The difference between the two lies, not, as previously believed, in the difference between the heat stabilities of the antibodies, but in the different competitive effects of unchanged antibody and the " mixed complexes." The precipitating or agglutinating titre of a heated antiserum is a function of this competition, and this is governed by the type of antigen. With anisodimensional antigens, i.e. when the flocculation corresponds to the type " H," the competing action of " mixed complexes " is slight, and the titre of heated antiserum is an approximate measure of antibody which remains unchanged after heating. But with antigens like bushy stunt virus, tobacco necrosis virus, blood serum proteins and bacteria giving " O " type floccules, the " mixed complexes " interfere effectively with the flocculating action of unchanged antibodies and the titre is not a direct measure of the amount of these.

With " O " antigens there are two causes leading to a fall in flocculating titre of antisera after heating, the transformation of antibodies into a form unable to flocculate and the ability of the transformed antibodies to interfere with the flocculating action of unchanged antibodies. With " H " type of antigens, however, the fall in flocculating titre results almost entirely from the first cause. As most tests on heating antisera have been made with " O " type antigens, it is obvious that published work on rate of inactivation of antisera by heat (Streng, 1909 ; Madsen and Streng, 1910), in which agglutinating titres have been used as a direct measure of antibodies remaining unchanged, needs reconsidering. The use of flocculating titres for this purpose has been responsible for the belief that antibodies to different antigens differed in their resistance to heat, and led Marrack (1938) to suggest that " the destruction of some antibodies must be associated with earliest degrees of heat denaturation of proteins, while others can resist even complete denaturation." All the antibodies heated in this work behaved similarly, and the apparent differences

in the heat stability of the antisera resulted from differences in the competitive action of the " mixed complexes."

Why " mixed complexes " fail to flocculate the antigen, although they combine with it, is unknown. The amount and the properties of the material forming complexes with antibody particles during heating, however, probably determine this. If, like albumin, it readily forms stable suspensions, union with antigen is less likely to give an insoluble product than if, like heated globulin, it forms unstable suspensions. Similarly, the formation of complexes composed largely of serologically unspecific material can change the behaviour of proteins which function as antigens (Bawden and Kleczkowski, 1941).

The formation of serologically active antibody-complexes occurs only over a limited range of heating, further heating leading to a loss of all serological activity. This applies equally to both " O " and " H " type of antigens. This change is reflected in the drop of flocculating titre of heated euglobulin solutions and in the disappearance of inhibition in solutions of " mixed complexes." This stage probably corresponds with a further stage of protein denaturation, in which the antibody particles are so altered that they no longer contain specific groups capable of combining with antigen.

SUMMARY.

Experiments on the effect of heat on rabbit antisera to the following antigens have been made: human serum albumin and globulin, a strain of pea nodule bacteria (*Rhizobium leguminosarum*); and purified preparations of the following plant viruses—tobacco mosaic, potato " X," tomato bushy stunt and tobacco necrosis.

Antisera to the rod-shaped viruses (tobacco mosaic and potato " X ") behave like those to flagellar type antigens, whereas antisera to the other antigens named behave like those to somatic type antigens, much more heating being needed to destroy the flocculating power of the former. However, euglobulin fractions (containing antibodies) of all the antisera behave similarly, and they require more heat to destroy their flocculating power than do the original antisera.

Flocculating antibodies undergo at least two changes during heating. Complexes composed of antibody particles and of particles of other unspecific proteins present in the solution are first formed. Antibodies changed in this way can still combine specifically with antigens, but the result of this combination depends on the quantity and quality of unspecific proteins present in the solution during heating. Complexes formed when antibodies are heated in the presence of euglobulin fraction of the antiserum flocculate their antigens. Complexes formed when antibodies are heated in the presence of other serum fractions, notably albumin, cannot flocculate their antigens, although they combine with them; this combination interferes with the flocculating action of antibodies that are unchanged. The degree of this interference depends on the type of antigen, being large with antigens of type " O " and small with those of type " H." This fact, and not a difference in heat stability, explains the differences in the behaviour of heated antisera to the two types of antigen.

The second change of antibodies during heating corresponds with a further stage of denaturation and is shown by the loss of ability to combine with antigen.

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SOME PROPERTIES OF COMPLEXES FORMED WHEN ANTIGENS ARE HEATED IN THE PRESENCE OF SEROLOGICALLY UNSPECIFIC PROTEINS.

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Received for publication June 20th, 1941.

KLECZKOWSKI (1941*b*, *c*) has presented evidence suggesting that rabbit antibodies can combine with other proteins while undergoing denaturation by heat. The serological behaviour of the complexes so formed depends on the protein with which the antibody has combined and on the type of antigen. Antibody-euglobulin complexes behave much like unchanged antibody, whereas antibody-albumin complexes can combine with, but not flocculate their antigens. Flagellar type antigens are much more readily flocculated by mixtures containing both unchanged antibody and antibody-albumin complexes than somatic type antigens. Because of this and not because of a greater resistance to heat, as previously believed, antisera to flagellar type antigens can be heated more than antisera to somatic type antigens without losing their ability to cause flocculation.

This paper shows that the serological behaviour of antigens after heating depends in the same way on what other proteins are present during heating and on whether the antigen is of the flagellar or somatic type. The antigens used were tomato bushy stunt and tobacco mosaic viruses and human serum

albumin and globulin. Tobacco mosaic virus is an antigen of the flagellar type giving a loose nebulous precipitate, and the others are of the somatic type, giving dense granular precipitates. The antigens and antisera were prepared and tested by the methods previously described.

The effects of heating tomato bushy stunt virus in the presence and absence of albumin.

When 0·05 per cent. solutions of tomato bushy stunt virus in saline at pH 7 are heated for 10 minutes at 80° C. they become opalescent, and a small sediment can be deposited by centrifuging at 8000 r.p.m., leaving an opalescent supernatant fluid. At 83° C. the fluids become increasingly opalescent, and although no floccules separate on standing, most of the material sediments on centrifuging to leave a water-clear supernatant fluid. Heating at 86° C. or higher causes the protein to settle out spontaneously. When the virus solution is heated in the presence of 0·5 per cent. rabbit-serum albumin it behaves

TABLE I.—*Effect of Heating Bushy Stunt Virus Solutions in the Presence and Absence of Rabbit Serum Albumin.*

0·05 per cent. solutions of bushy stunt virus in physiological saline and in 0·5 per cent. rabbit serum albumin solutions were heated for 10 minutes at different temperatures at pH 7·0.

1 ml. of the bushy stunt virus preparations diluted 1/10 in saline were added to a series of tubes each containing 1 ml. of varying dilutions of a bushy stunt virus antiserum.

+ indicates precipitation and − no precipitation.

After 3 hours 0·1 ml. of 0·05 per cent. solution of control bushy stunt virus was added to the tubes containing no precipitate.

i indicates that there was still no precipitation (inhibition) and o indicates the separation of precipitate (no inhibition).

Temperature of heating.	Solvent.	Dilution of the antiserum. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
Unheated	Saline	+	+	+	+	−	−
80° C.	,,	+	+	+	+	+	−
,,	Albumin solution	−	−	−	−	−	−
		o	i	i	i		
83° C.	Saline	+	+	+	+	+	+
,,	Albumin solution	−	−	−	−	−	−
		i	i	i	i		
90° C.	,, ,,	−	−	−	−	−	−
		i	i	i	i		
95° C.	,, ,,	−	−	−	−	−	−
		o	o	o	o		
	Solutions of bushy stunt virus and of rabbit albumin in saline mixed after being heated separately.						
80° C.	..	+	+	+	+	+	−
83° C.	..	+	+	+	+	+	+

quite differently. Solutions heated at 80° and 83° do not become opalescent, and no precipitate separates even after heating at 95° C., although with this treatment the fluid becomes highly viscous and opalescent.

The infectivity of the virus solutions is destroyed by 10 minutes heating at 80° C., both in the presence and absence of albumin, but the ability to combine with virus-antibody is retained. The serological behaviour of the heated solutions is shown in Table I. The stable opalescent suspensions produced by heating the virus in saline at 80° C. and 83° C. are flocculated specifically by virus-antiserum. Floccules form sooner and the suspensions are flocculated by higher dilutions of antiserum than solutions with the same concentration of unheated virus. By contrast, no flocculation occurs when preparations of bushy stunt virus heated at between 80° C. and 90° C. in the presence of albumin are added to virus-antiserum. Although there is no flocculation, the heated virus-albumin mixture does combine with the virus-antibody, for it inhibits flocculation when unheated virus is added subsequently. In other words, heating the virus in the presence of albumin has produced something behaving like a non-precipitating hapten, able to combine with antibody and to prevent it from flocculating unchanged virus.

Heating for 10 minutes at 95° C. in the presence of albumin destroys the ability to combine with virus-antibody, for when such preparations are tested against virus antiserum they fail to flocculate or to inhibit the flocculation of unheated virus added subsequently. Inhibition only occurs when the virus and albumin are heated together at temperatures between 80 and 90° C. If the two are heated separately and then mixed, the mixtures flocculate with antiserum in the same way as heated virus in the absence of albumin. Thus the non-precipitating hapten is formed only when the virus and albumin are heated together over a limited range of temperature. For the inhibition to be complete there must also be considerably more albumin in the heated fluids than virus. In Table I where inhibition is complete, the ratio of albumin to virus was 10 to 1, but with a ratio of 4 to 1 flocculation with antiserum still occurs, although much more slowly than with unheated virus.

Table II shows that the material rendered sedimentable by heating solutions of bushy stunt virus in saline for 10 minutes at 80° and 83° C. is still flocculated by virus-antiserum. This was shown by taking up the sedimented material in saline, in which it formed stable suspensions, and testing against virus-antiserum, which flocculated it specifically. The denatured protein that separates spontaneously from virus solutions heated at 86° C. does not resuspend to give stable suspensions, but if sufficiently diluted it remains stable long enough to demonstrate that it is flocculated specifically by virus-antiserum. The material separating at 90° and higher is too unstable for such tests, for it flocculates rapidly in the presence of normal rabbit serum.

The ability of the precipitates formed by heating bushy stunt virus at different temperatures to combine with antibody was tested in another way. Suspensions of the heated virus were mixed with antiserum at various dilutions, incubated for 3 hours at 50° C., and the precipitates removed by centrifugation. The supernatant fluids were then tested for their antibody content by the addition of unheated virus. The unabsorbed serum reacted at a dilution of

TABLE II.—*Effect of Heating Bushy Stunt Virus Solution in Physiological Saline.*

0·05 per cent. solutions of bushy stunt virus in saline at pH 7·0 were heated for 10 minutes at different temperatures. The heated suspensions (if stable), the supernatant fluids obtained after centrifuging at 8000 r.p.m. and the precipitates resuspended in the original volume of saline were used for serological tests.

First test.

1 ml. of each preparation of bushy stunt virus at a dilution of 1/10 in saline was added to a series of tubes each containing 1 ml. of varying dilution of antiserum.

+ indicates flocculation and − no flocculation.

Second test.

After 3 hours the contents of the tubes were centrifuged and 0·1 ml. of 0·05 per cent. virus solution was added to the supernatant fluids.

+ indicates flocculation (presence of unabsorbed antibodies) and − indicates no flocculation.

Temperature of heating.	Preparation of bushy stunt virus.	Dilutions of antiserum. First test. 1/10.	1/20.	1/40.	1/80.	1/160	1/320.	Second test. 1/10.	1/20.	1/40.	1/80.
Unheated	Solution	+	+	+	+	−	−	+	−	−	−
80° C.	Orig. suspension	+	+	+	+	+	−				
,,	Supernatant	+	+	+	+	+	−				
,,	Precipitate	−	−	−	+	+	+				
83° C.	Orig. suspension	+	+	+	+	+	+	+	+	−	−
,,	Supernatant	−	−	−	−	−	−				
,,	Precipitate	+	+	+	+	+	+	+	+	−	−
86° C.	Supernatant	−	−	−	−	−	−				
,,	Precipitate ½ hr.	−	−	+	+	+	+				
	1 hr.	+*	+*	+*	+*	+*	+*	+	+	−	−
90° C.	,, ½ hr.	+*	+*	+*	+*	+*	+*	+	+	+	−
95° C.	,, ½ hr.	+*	+*	+*	+*	+*	+*	+	+	+	+

* Unspecific flocculation due to instability of the suspension and formed also in the controls with normal rabbit-serum.

1/80 and after absorption with unheated virus it reacted at 1/10. The precipitation end-point after absorption with material precipitated at 83°, 86°, 90°, and 95° was 1/20, 1/20, 1/40, and 1/80 respectively. Thus the virus precipitated by heating at 83° and 86° combined with approximately half as much antibody as the same amount of unheated virus and that heated at 90° C. with a quarter as much, whereas virus heated at 95° C. combined with no antibody. The amount of heating required to denature and precipitate bushy stunt virus, therefore, is less than that required to destroy its ability to combine

with antibody, and this is apparently the same whether the virus is heated alone or in the presence of albumin.

Four changes can be detected in bushy stunt virus preparations heated for 10 minutes at different temperatures. Below 80° C. there is loss of infectivity, with no change in the serological reactions, and at 95° C. and higher the virus loses its ability to combine with its antibody. The serological behaviour after heating at temperatures between 80° C. and 95° C. depends on whether the virus is heated alone or with albumin. If heated in saline around 83° C. the solutions become opalescent and are more easily flocculated by antiserum, whereas at 86° C. or higher the virus becomes insoluble, though still able to combine with antibody. When heated with albumin in this range the virus remains in suspension and able to combine with antibody, but is not flocculated by the combination.

The effects of heating tobacco mosaic virus in the presence and absence of albumin.

When tobacco mosaic virus antiserum is heated at temperatures around 80° C. it continues to flocculate its antigen whereas bushy stunt virus antiserum does not (Kleczkowski, 1941*c*). Similarly, the serological behaviour of the two viruses differs when they are heated in the presence of albumin, for although tobacco mosaic virus interacts with albumin, the product of heating does not behave like a non-precipitating hapten. The appearance of heated preparations of tobacco mosaic virus depends on the pH and on whether albumin is present. 0·05 per cent. solutions of virus in *M*/15 phosphate buffer at pH 6 become slightly opalescent after 10 minutes at 80° C., and still show the phenomenon of anisotropy of flow strongly, whereas at pH 7 much of the material separates as a precipitate and anisotropy of flow disappears. After 10 minutes at 90° C. precipitates separate at pH 6 as well as pH 7, and anisotropy of flow is lost. When 0·05 per cent. virus solutions are heated in the presence of 0·6 per cent. rabbit serum albumin at pH 7, no precipitates separate after 10 minutes heating at 80° C. or 90° C. After heating at 80° C. there is some increased opalescence and the anisotropy of flow is reduced, though not destroyed, and at 90° C. the fluids become extremely opalescent and viscous and no anisotropy of flow can be detected.

Table III shows the effects on the infectivity and serological activity of heating tobacco mosaic virus for 10 minutes at 80° and 90° C. under different conditions. The conditions under which the fluids are heated at 80° C. greatly affect the activity of the virus. At pH 7 both infectivity and serological activity are reduced to about one-twentieth of the control, and the residual activity is in the remaining soluble material. At pH 6 there is no loss of serological activity and a small drop in infectivity. The presence of albumin in the virus solutions heated at pH 7 protects them almost completely from inactivation. The mere addition of albumin, either heated or unheated, to the virus solutions causes a reduction in infectivity, the reduction being much less apparent after diluting the mixtures, but there is little further loss when the mixtures are heated for 10 minutes at 80° C., and only a small drop in serological activity. Heating for 10 minutes at 90° C. has destroyed both infectivity and

TABLE III.—*Effect of Heating Tobacco Mosaic Virus Solutions in Different Conditions.*

0·05 per cent. virus solutions were heated for 10 minutes in saline at pH 7·0, in phosphate buffer at pH 6·0 and in 0·6 per cent. rabbit serum albumin solution at pH 7·0.

Prep. no.	Temperature of heating.	pH.	Diluent.	Serological titre of the preparation.	Average number of lesions per leaf at 1/1.	Average number of lesions per leaf at 1/20.
1	Unheated	7·0	Saline	1/160	115	53
2	80° C.	6·0	Phosph. buffer	1/160	103	37
3	,,	7·0	Saline	..	27	10
4	Supernatant from (3)			1/8	35	11
5	Resuspended precipitate from (3)			..	0·5	0
6	80° C.	7·0	Albumin solution	1/120	21	28
7	Unheated	7·0	,, ,, heated at 80° C.	1/160	32	34
8	,,	7·0	Unheated albumin solution	1/160	26	29
9	90° C.	6·0	Phosph. buffer	..	0	0
10	Supernatant from (9)			No pptt. at 1/2.		
11	90° C.	7·0	Saline	..	0	0
12	Supernatant from (11)			No pptt. at 1/2.		
13	90° C.	7·0	Albumin solution	,, ,,	0	0

the serological reactions. The ability to combine with antibody was destroyed both by heating alone and in the presence of albumin, for incubating antiserum with the material precipitated by heat did not reduce its antibody content, and incubation with the heated virus-albumin mixture did not prevent it from flocculating active virus added subsequently.

The disruption by heat and loss of all specific properties seems to occur more suddenly with tobacco mosaic virus than with bushy stunt virus, and fewer successive changes can be detected on heating. At pH 6 there is a suggestion that infectivity is being lost before denaturation or loss of serological activity occurs, and similar separations of these two activities have been described earlier (Lauffer and Price, 1940 ; Bawden and Pirie, 1940), but the separation is slight compared with bushy stunt virus. The protein that separates from denatured tobacco mosaic virus preparations is free from nucleic acid, whereas that from bushy stunt virus contains nucleic acid, suggesting that the nucleic acid-protein linkage is more stable in bushy stunt than in tobacco mosaic virus. This may explain why the precipitates formed on heating bushy stunt virus still combine with virus-antibody whereas those from tobacco mosaic virus do not, for it may be that the nucleic acid-protein link is necessary for serological specificity. Alternatively, it may be that the breaking of such a linkage is followed immediately by the loss of all structural specificity, whereas in bushy stunt virus other, structurally less important linkages are more sensitive to heating, and the breaking of these one-by-one is responsible for each stage that can be detected in the loss of specific activities on heating.

The effect of albumin in protecting tobacco mosaic virus against heat-inactivation suggests that denaturation may be in part a reversible reaction. In the absence of albumin the end-product is insoluble and is removed from the reaction, which can therefore rapidly proceed to completion. But in the presence of albumin there is no precipitation and this may slow down the reaction.

The effects of heat on the serological reactions of human serum proteins.

Previous work on the influence of heat on the antigenic specificity of animal sera has given conflicting results, for some workers have stated that heating destroys all the original specificity whereas others have stated that it merely causes modifications (e.g. Obermayer and Pick, 1904 ; Furth 1925). Spiegel-Adolf (1926) showed that heated serum could combine with its antibody without being precipitated and that this combination prevented precipitation when unheated serum was added later. Antibody made in one animal by the injection of another animal's serum is often predominantly to globulin (Kleczkowski, 1938), and albumin then represents a serologically unspecific protein. The conflicting results may therefore arise from the fact that the antisera used by different workers varied in their content of antibody to globulin and albumin, for our results show that the serological behaviour of heated antigenic proteins is greatly modified by the presence of serologically unspecific proteins during heating.

Table IV shows the results of precipitin tests with 0·05 per cent. solutions of whole globulin from human serum after ten minutes' heating at 80° C. in saline and in 0·5 per cent. solutions of rabbit serum albumin and human albumin. After heating in saline the globulin is still precipitated by its antiserum, but after heating in the presence of either of the albumins there is no precipitation and the presence of the heated proteins prevents the precipitation of unheated globulin added later. The heated mixture of globulin and human albumin, however, was still precipitated by albumin antiserum.

When human albumin is heated at 80° C. in the presence of rabbit serum albumin its serological behaviour is altered so that it combines with antibody but is not precipitated by it (Table VI). As with globulin-albumin mixtures the ability to inhibit the precipitation of unheated antigen is developed only when the serologically specific and unspecific proteins are heated together and not when they are heated separately and then mixed.

When heated at 80° C. in the presence of serologically unspecific albumin, human globulin and albumin differ in their serological behaviour from tobacco mosaic virus and resemble bushy stunt virus, i.e. all the somatic type antigens tested behave in the same way. Their serologically active groups, however, are more resistant to heat than those of bushy stunt virus, for human albumin and globulin continue to precipitate with their antisera after heating separately for 5 minutes at 100° C. (Tables V and VII). Similarly, when heated at this temperature in the presence of twice their concentration of serologically unspecific protein, both human albumin and globulin continue to combine with their antibodies and to behave like non-precipitating haptens.

TABLE IV.—*Effect of Heating for* 10 *Minutes at* 80° *C. Human Serum Globulin Solution alone and in the Presence of Human or Rabbit Serum Albumin.*

0·05 per cent. solutions of human rabbit serum globulin were heated in saline and in 0·5 per cent. human or rabbit serum albumin solutions.

1 ml. of an antiserum to human serum globulin diluted 1/20 was added to a series of tubes each containing 1 ml. of varying dilutions of human globulin.

+ indicates precipitation and − no precipitation.

After 3 hours 0·1 ml. of 0·06 per cent. solution of human serum golbulin was added to the tubes in which there was no precipitation.

i indicates that there was still no precipitation (inhibition) and o indicates the separation of precipitate (no inhibition).

Preparation of human globulin.	Amount of human globulin (in mg.).				
	0·25	0·12.	0·06.	0·03.	0·015.
Unheated solution in saline . .	+	+	+	+	+
Heated solution in saline . .	+	+	+	+	−
Heated solution with human albumin*	−	−	−	−	−
	i	i	o	o	o
Heated solution with rabbit albumin	−	−	−	−	−
	i	i	o	o	o
Solution in saline mixed with human albumin solution after heating separately	+	+	+	+	−
					o
Solution in saline mixed with rabbit albumin solution after heating separately	+	+	+	+	−
					o

* This preparation precipitated with antiserum to human serum albumin.

TABLE V.—*Effect of Heating for* 5 *Minutes at* 100° *C. Human Serum Globulin Solutions alone and in the Presence of Human Albumin.*

0·1 per cent. solutions of human serum globulin were heated in saline and in 0·2 per cent. human serum albumin solution.

The antiserum to human serum globulin was used at a dilution of 1/40, otherwise the methods of testing were as described for Table IV.

Preparation of human globulin.	Amount of human globulin (in mg.).					
	0·5.	0·25.	0·12.	0·06.	0·03.	0·015.
Unheated solution . . .	+	+	+	+	+	+
Heated solution in saline . .	+	+	+	+	+	−
Heated solution with human albumin	−	−	−	−	−	−
	i	i	o	o	o	o
Solution in saline mixed with human albumin solution heated separately	+	+	+	+	+	−
						o

TABLE VI.—*Effect of Heating for* 10 *Minutes at* 80° *C. Human Serum Albumin Solutions alone and in the Presence of Rabbit Serum Albumin.*

0·05 per cent. solutions of human serum albumin were heated in saline and in 0·5 per cent. rabbit serum albumin solution.

1 ml. of an antiserum to human serum albumin diluted 1/40 was added to a series of tubes each containing 1 ml. of varying dilutions of human serum albumin. After 3 hours 0·1 ml. of 0·06 per cent. solution of human serum albumin was added to the tubes where there was no precipitation.

Preparation of human albumin.	Amount of human albumin (in mg.).			
	0·06.	0·03.	0·015.	0·0075.
Unheated solution	+	+	+	+
Heated solution in saline	+.	+	+	+
,, ,, with rabbit albumin	—	—	—	—
	i	i	i	i
Solution in saline mixed with rabbit albumin solution after heating separately	+	+	+	+
Heated 0·5 per cent. human albumin solution in saline	+	+	+	+

TABLE VII.—*Effect of Heating for* 5 *Minutes at* 100° *C. Human Serum Albumin Solutions alone and in the Presence of Rabbit Serum Albumin.*

0·1 per cent. solutions of human serum albumin were heated in saline and in 0·2 per cent. rabbit serum albumin solution.

Methods were as in Table VI.

Preparation of human albumin.	Amount of human albumin (in mg.).			
	0·06.	0·03.	0·015.	0·0075.
Unheated solution	+	+	+	+
Heated solution in saline	+	+	+	+
,, ,, with rabbit albumin	—	—	—	—
	i	i	o	o
Solution in saline mixed with rabbit albumin solution after heating separately	+	+	+	+

The precipitation of viruses heated with albumin by human albumin antiserum.

The most reasonable explanation of the change in the serological behaviour of somatic type antigens after heating in the presence and absence of albumin is that the antigens combine with other proteins while undergoing heat denaturation to produce complexes, which remain soluble after uniting with antibody. Positive evidence for such a combination between bushy stunt virus and human albumin was supplied when it was found that the virus, heated in the presence of human albumin, could be precipitated by human albumin antiserum. 0·05 per

cent. solutions of bushy stunt virus in saline and in 0·6 per cent. solution of human albumin were heated for 10 minutes at 80° C. The virus heated in saline was precipitable by virus antiserum, whereas that heated with albumin was not, and inhibited the precipitation of unheated virus, although it precipitated with antiserum to human albumin. Antiserum to human albumin was allowed to react at the constant serum optimal proportions with the virus and albumin heated together and with a mixture of the same proportions of the two heated separately. After two hours at 37° C. and 48 hours at 1° C., the fluids were centrifuged and the supernatant fluids tested against antiserum to bushy stunt virus. The virus and albumin heated together now neither precipitated with virus antiserum nor inhibited the precipitaton of unheated virus added later, whereas the mixture of virus and albumin heated separately still precipitated with virus antiserum to the same extent as before it was absorbed with human albumin antiserum.

As the precipitation and the inactivation of tobacco mosaic virus by heat at pH 7 is retarded by the presence of albumin, it is obvious that there is an interaction between the two. An experiment similar to that described with bushy stunt virus showed that tobacco mosaic virus also combines with albumin when the two are heated together. 0·05 per cent. solutions of tobacco mosaic virus were heated for 10 minutes at 80° C. in the presence of 0·6 per cent. human albumin, and samples were allowed to react fully with antiserum to human albumin. Before reacting with this serum the heated mixture of virus and albumin precipitated strongly with virus antiserum, but after absorption with human albumin antiserum it did not. A mixture of unheated virus and heated albumin, however, precipitated with virus antiserum equally before and after absorption with antiserum to human albumin. Thus with both viruses it is only when they have been heated in the presence of the albumin that they can be removed by absorption with human albumin antiserum.

DISCUSSION.

Evidence that serum proteins can combine when heated together has recently come from a number of sources. Kleczkowski (1941*b*) has shown that a new component readily sedimentable appears in heated rabbit serum, and Van der Scheer *et al.* (1941) and Krejci *et al.* (1941) have shown by electrophoretic studies that new components appear in horse serum, both normal and anti-pneumococcal, after heating. The results described in this paper show that other proteins, as different as plant viruses and serum proteins, can also combine when undergoing heat denaturation together. The complexes formed may have different physical properties from either of the two components heated separately. For example, when euglobulin is heated with albumin there is no spontaneous precipitate of denatured protein, but a material is produced which is deposited as a gel on moderate centrifugation and can be dispersed in water to give a stable suspension. Similarly, when bushy stunt and tobacco mosaic viruses are heated with albumin they give no such precipitates as when heated alone, but the complexes they form with albumin must be smaller than that formed by globulin, for nothing can be sedimented from the heated mixtures of viruses and albumin by centrifuging at 8000 r.p.m.

It has previously been suggested (Steinhardt, 1938 ; Bawden and Pirie, 1940) that the efficiency of denaturating agents depends on their ability to break cross linkages between peptide chains in proteins, and that the probability of the linkages returning to their original pattern on the removal of the agent is lessened by the increase in the number of such linkages broken. If denaturation by heat is also assumed to result from breakage of cross linkages, our results can be explained on the basis that the linkages are reformed purely at random, and that when more than one protein is being heated cross linkages are as likely to reform between chains in different proteins as between chains in the same protein. If this is so, then it is likely that complexes will only be formed between proteins in which similar cross linkages are being broken at the same time, and that those that denature at widely different temperatures will not combine in the same way. All the proteins used in this work undergo demonstrable changes when heated alone for a few minutes around 80° C., which is the amount of heating required to produce complexes when they were heated together.

Although it is possible that combination can occur between other proteins possessing similar heat stabilities, the effects on the serological reactions of the proteins might be expected to differ from those described here. All the serological changes were produced by heating antigens in the presence of serologically unspecific albumin and tended to make the antigen less precipitable by its antibody. Heated albumin is very stable and combination between antigens and other, less stable proteins might be expected to give different results.

The fact that tobacco mosaic virus after heating with albumin still flocculates with its antiserum, whereas somatic types antigen do not, cannot be attributed to a failure of this virus to unite with albumin, for that combination does occur is shown by the reduction in the rate of inactivation by heat, by the fact that no spontaneous precipitate of denatured protein separates, and by the precipitation of virus heated with albumin by albumin antiserum. Tobacco mosaic virus is more readily flocculated by its antibody than antigens of the somatic type ; floccules separate quicker, and they are produced with less antibody and over a much wider range of antigen/antibody ratios. (Kleczkowski, 1941*a*). This greater insolubility of the tobacco mosaic virus-antibody combination may explain the differences in the serological behaviour of flagellar and somatic type antigens after heating with albumin. A similar difference is found when antisera to the two kinds of antigen are heated, for after the same amount of heating those to somatic type antigens do not cause flocculation but those to flagellar type antigens do. When the ratio of albumin to antibody is increased by about three times that in serum, however, heating causes antibodies to tobacco mosaic virus to behave like those to somatic type antigens and to inhibit flocculation. Thus it seems that combination of either somatic type antigens or their antibodies with relatively small amounts of soluble, serologically unspecific protein increases the solubility of the complex formed by the union of antigen and antibody sufficiently to prevent flocculation, whereas a complex containing much more unspecific protein is necessary to keep the combination of flagellar type antigen and antibody in solution.

SUMMARY.

When tomato bushy stunt virus, human serum globulin and albumin are heated at temperatures around 80° C. in the absence of serologically unspecific proteins, they are still able to precipitate with their specific antisera. If heated in the presence of serologically unspecific serum albumin, however, they produce complexes behaving like non-precipitating haptens. Human globulin, and albumin behave in this way after being heated for 5 minutes at 100° C., but bushy stunt virus does not combine with antibody after heating for 10 minutes at 95° C.

The presence of albumin in solutions of tobacco mosaic virus heated at pH 7·0 reduces the rate of inactivation, but this flagellar type antigen still flocculates with its antiserum. This virus loses its ability to combine with antibody after 10 minutes at 90° C.

Heating either virus in the presence of albumin prevents the separation of precipitates of denatured protein, and that combination between the viruses and albumin occurs is shown by the fact that the viruses can then be removed by precipitation with antiserum to the albumin.

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A SIMPLIFIED METHOD FOR THE ESTIMATION OF SULPHANILAMIDE AND SULPHAPYRIDINE IN BIOLOGICAL FLUIDS.

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Received for publication June 25, 1941.

THE extensive use of M.&B. 693 or sulphanilamide treatment, and the recognized importance of maintaining an optimal body fluid concentration, make it eminently desirable to have a method which combines simplicity and rapidity in estimating these chemotherapeutic agents. By making use of the principle utilized by Scott (1941) that azobilirubin can be quantitatively determined in blood serum without precipitation of the blood proteins, accurate means can be devised for the determination of 693 or sulphanilamide in biological fluids rather more simply than has hitherto been possible by the methods now in vogue. The convenience of the method described below is such that it is possible to obtain estimations of the sulphonamide group in cerebro-spinal fluid or blood serum without first removing the proteins by trichloracetic acid or paratoluene sulphonic acid. The procedure depends on the careful adjustment of the pH and the alcoholic concentration governing the diazotization process of Marshall (1937), which employs alcoholic dimethyl-alpha-naphthylamine for the production of the azo dye from sulphonamide compounds. Colorimetric comparison is made against a suitable standard, i.e., dilute aqueous solutions of 693 or para-aminobenzene-sulphonamide (sulphanilamide), with selected amounts of normal horse serum added as a standard protein which is subjected to exactly the same treatment as the test fluid. A specially revised method is provided for the determination of sulphonamide in whole blood and lipaemic or haemolysed sera, when for various reasons it is impossible to obtain pure serum for the test. In the process about to be outlined here, no micro-quantities are handled and no delicate or refined manipulation is required. The solutions themselves are few in number and simple in preparation.

METHODS.

Reagents.

1. (*a*) Stock standard of M.&B. 693 Dagenan. 100 mg. of pure 693 dissolved in 100 c.c. of N/100 sodium hydroxide. A special preparation of 693 is supplied for chemical analysis by May & Baker, Ltd., Dagenham.

(*b*) Stock standard of sulphanilamide, 100 mg. of para-aminobenzene-sulphonamide (B.D.H.) per 100 c.c. of water.

2. Standard solutions of 693 and sulphanilamide. Two standards are

prepared of each, 2·5 and 5 c.c. being diluted to 100 c.c. with water. 1 c.c. equals 0·025 and 0·05 mg. of 693 and sulphanilamide respectively.

3. Three per cent. (by volume) hydrochloric acid.

4. One in 10 dilution with water of 0·5 per cent. aqueous sodium nitrite solution. (The 1 in 10 dilution is prepared prior to the test or freshly each day.)

5. Fifty per cent. aqueous solution of urea.

6. One per cent. (by volume) alcoholic solution of dimethyl-alpha-naphthylamine. Prepared freshly each month from stock solution (B.D.H.), and stored in an amber ground glass stoppered bottle.

7. Diluent for colorimetry : 20 c.c. of water ; 5 c.c. of 3 per cent. hydrochloric acid ; 5 c.c. of absolute alcohol.

8. Normal horse serum and oxalated whole blood (Burroughs Wellcome & Co.).

9. Approximately 0·25 per cent. protein solution prepared by a 1 in 25 dilution of the horse serum with 0·5 per cent. sodium chloride. Five to 6 drops of 10 per cent. alcoholic thymol solution is added as preservative.

10. Cordite tubes of uniform bore, graduated in $\frac{1}{10}$ c.c. and of 5 c.c. capacity. (Diameter 1 cm., length 12·5 cm.)

Note.—Solutions 1, 2, 6, 8, 9 should be kept in the refrigerator and are then stable indefinitely.

Procedure for Cerebro-spinal Fluid.

Introduce into two test tubes (graduated at 5 c.c.) in the following order, with thorough mixing after each addition :

Standard.	Test C.S.F.
0·5 c.c. of standard (0·05 mg. per c.c.) . .	1 c.c. of C.S.F.
0·5 c.c. of 0·25 per cent. protein solution . .	0·5 c.c. of 3 per cent. hydrochloric acid.
0·5 c.c. of 3 per cent. hydrochloric acid	
0·5 c.c. of 1 in 10 dilution of sodium nitrite .	0·5 c.c. of 1 in 10 dilution of sodium nitrite.

Stand at room temperature for 5 minutes.

Add 0·5 c.c. of 50 per cent. urea solution to each tube, mix well, and stand a further 3 to 5 minutes for the urea to destroy excess of sodium nitrite. (The unused residual nitrous acid otherwise reacts with the dimethyl-a-naphthylamine to form a brownish-orange coloration that makes colorimetric comparison somewhat difficult.)

Add 0·5 c.c. of 1 per cent. alcoholic dimethyl-a-naphthylamine to the contents of each tube and mix very thoroughly. Stand 10 minutes, and then dilute both volumes to the 5 c.c. mark with the special diluent fluid. Mix again, and compare colorimetrically or by dilution colorimetry.

Calculation.—$\frac{S}{U} \times 0{\cdot}025$ mg. $\times$ 100 equals mg. of sulphonamide per 100 c.c. of C.S.F.

Procedure for Blood Serum.

Standard.	Test serum.*
0·5 c.c. of normal horse serum . . .	0·5 c.c. of serum.
0·5 c.c. of standard (0·025 or 0·05 mg. per c.c.) .	0·5 c.c. of water.
0·5 c.c. of 3 per cent. hydrochloric acid . .	0·5 c.c. of 3 per cent. hydrochloric acid.
0·5 c.c. of 1 in 10 dilution of sodium nitrite .	0·5 c.c. of 1 in 10 dilution of sodium nitrite.

Stand at room temperature for 5 minutes.

* It should be noted that the occurrence of haemolysis in a serum renders the above technique useless, it being impossible to match the colours of standard and test solution.

Add 0·5 c.c. of 50 per cent. urea solution to each tube, stand again for 3 to 5 minutes, and then add 0·5 c.c. of dimethyl-a-naphthylamine to the contents of both tubes. Mix and stand 10 minutes, after which the volumes are diluted to 5 c.c. with the dilution fluid. Mix thoroughly and compare in the colorimeter or by colorimetry.

Calculation.—$\frac{S}{U} \times 0{\cdot}0125$ or 0·025 mg. $\times$ 200 equals mg. of sulphonamide per 100 c.c. of serum.

Procedure for whole blood and lipaemic or haemolysed sera.

Into a small test tube introduce 2 c.c. of water, add 1 c.c. of whole blood, serum or plasma. After mixing well, stand 2 to 3 minutes for plasmolysis to take place. Add 2 c.c. of 15 per cent. trichloracetic acid drop by drop with constant shaking. Finally the mixture is vigorously shaken and filtered through a No. 1 5·5 cm. Whatman paper. 2·5 c.c. of filtrate is pipetted into a 5 c.c. graduated test tube and 0·5 c.c. of 1 in 10 dilution of sodium nitrite added. The tube is stood for five minutes at room temperature and then 0·5 c.c. of urea solution added followed by 0·5 c.c. of alcoholic dimethyl-a-naphthylamine.

The standard is prepared at the same time by adding 0·25 c.c. (with a micro-pipette) of stock sulphonamide solution (100 mg. per 100 c.c.) to 4·75 c.c. of oxalated horse blood or horse serum as the case may be. Mix thoroughly and pipette 1 c.c. into 2 c.c. of water. The above procedure for the test blood is then followed in detail.

After the addition of the coupling reagent to the standard and test solutions, the colours are allowed to develop at room temperature for 15 minutes and then diluted to 5 c.c. with the following diluent: 25 c.c. of water, 10 c.c. of 15 per cent. trichloracetic acid, 5 c.c. of absolute alcohol. The contents of each tube are mixed well and then comparison made by dilution colorimetry (using the diluent just described), or by colorimetric means.

Calculation.—$\frac{S}{U} \times 5$ equals mg. of sulphonamide per 100 c.c.

Estimation in Urine.

A 1 in 5 or 1 in 10 dilution of the urine is made with the 0·25 per cent. protein solution and 0·5 c.c. is taken and compared against a suitable standard.

Standard.	Urine.
0·5 c.c. of standard (0·025 or 0·050 mg. per c.c.)	0·5 of 1 in 5 or 1 in 10 dilution of the test urine with 0·25 per cent. protein solution.
0·5 c.c. of normal control urine diluted in the same proportion as the test urine with 0·25 per cent. protein solution	0·5 c.c. of water.

Then proceed as for the blood serum method.

Calculation.—$\frac{S}{U}$ × 0·0125 or 0·025 mg. × dilution of urine × 200 equals mg. of sulphonamide per 100 c.c. of urine.

With other body fluids such as transudates or exudates, treat exactly as for the blood serum method.

For general routine purposes the colorimetry method of comparison is to be preferred to the colorimetric procedure in that a much greater difference in colour depth between the standard and test solution can be accurately matched by reason of the standard bore of the cordite tubes and the uniform depth of colour comparison, that is, the colour is proportional over a wider range to the concentration of the sulphonamide present. The colorimetry calculation is the same as that recorded for the colorimetric technique, except that the volume of the test solution expressed in c.c. is divided by the standard volume in c.c.

A series of comparative results using the colorimetry and colorimetric methods on samples of cerebro-spinal fluid serum and whole blood taken from patients treated with sulphonamide is compiled in the table below. The figures represent mg. of free sulphonamide per 100 c.c. of body fluid.

TABLE I.

Nature of fluid.	Added sulphonamide.	Direct diazotization.		Trichloracetic acid method.	
		Colorimetry.	Colorimetric.	Colorimetry.	Colorimetric.
Meningitis C.S.F. (treated 693)	—	1·6	1·9	1·7	1·7
,, ,, ,,	—	4·0	4·1	4·1	4·2
,, ,, ,,	—	6·6	6·9	6·7	6·9
,, ,, ,,	—	11·0	11·6	10·8	11·4
Control meningitis C.S.F. before treatment	2·5	2·5	2·7	2·5	2·6
,, ,, ,, ,,	5·0	5·0	5·1	5·2	5·4
,, ,, ,, ,,	10·0	9·9	10·3	9·7	10·1
Blood-serum (treated 693)	—	2·2	2·4	2·2	2·4
,, ,,	—	5·6	5·8	5·7	5·6
,, ,,	—	10·4	10·8	10·5	9·9
Control serum (normal subject)	2·5	2·5	2·7	2·5	2·6
,, ,, ,, ,,	5·0	5·1	5·2	5·2	5·3
,, ,, ,, ,,	10·0	9·9	10·2	9·8	9·9
,, ,, ,, ,,	15·0	14·8	15·1	13·3	13·5
Oxalated whole blood (treated 693)	—	1·9	2·3	2·1	2·2
,, ,, ,,	—	5·0	5·5	5·3	5·3
,, ,, ,,	—	11·4	11·9	11·1	11·2
,, ,, ,, (normal)	5·0	5·0	5·1	5·0	5·0
,, ,, ,, ,,	10·0	10·0	10·3	9·9	10·1
,, ,, ,, ,,	15·0	14·7	15·1	13·3	13·4

It might be mentioned that the precipitation of the proteins of the cerebro-

spinal fluid is carried out as follows : 3 c.c. of water, 1 c.c. of C.S.F., 1 c.c. of 15 per cent. trichloracetic acid. After filtration through a 5·5 cm. paper the whole blood method is followed in detail.

The lower values obtained by the trichloracetic acid with whole blood after the addition of 15 mg. of sulphonamide per 100 c.c. demonstrates the necessity of employing a stronger standard (0·5 c.c. of stock standard plus 4·5 c.c. of oxalated horse blood) when the amounts of sulphonamide are much in excess of this figure.

After the determination of the sulphonamide content of blood serum, cerebro-spinal fluid or urine, the samples can be preserved indefinitely by the addition of 1 to 2 drops of 10 per cent. alcoholic thymol per 10 c.c. of body fluid. They are kept in the refrigerator, and may be employed as comparison standard against the biological fluids they represent, with a saving of time in the preparation of artificial standards.

FURTHER OBSERVATIONS.

The advantages of the direct diazotization method can now be stated in more detailed terms. Preliminary trials of the method described by Marshall (1937) for the determination of blood sulphonamide furnished evidence which seemed to make it desirable to modify it in certain directions. The first unsatisfactory feature is that the colour produced from pure standard solutions of sulphonamide do not compare favourably in colour tint with the coloration obtained from extracts of whole blood. When using whole blood precipitated with trichloracetic acid, the filtrate contains substances which interfere with the colour reaction between sulphonamide and dimethyl-a-naphthylamine, so that an inhibitory effect is observed with the development of colour in the unknown, which proceeds slower and is finally less intense than a control aqueous standard of sulphonamide similarly treated. It might be mentioned that the actual colour tone and rate of development of the azo-dye depends on certain factors, among which are the following : (1) the hydrogen ion concentration ; (2) the percentage of alcohol introduced with the dimethyl-a-naphthylamine ; (3) the presence or absence of sodium chloride ; (4) the presence or absence of the non-protein nitrogenous constituents ; (5) excess of nitrous acid from the diazo reaction.

The second drawback is the adsorption of a small amount of sulphonamide on the protein precipitate during the removal of the blood protein by trichloracetic acid. Although Marshall claims to have made a 100 per cent. recovery of sulphonamide from blood, the present writer has encountered losses of approximately 5 per cent. with serum and a greater adsorption power with whole blood, which sometimes reaches a maximum value of as much as 20 per cent. with samples rich in meta-protein. Again the manner in which the protein precipitate is formed has a direct relationship to the amount of sulphonamide adsorbed on the precipitated proteins, an increased loss being observed when whole blood is added to strong solutions of trichloracetic acid.

It is fundamental, therefore, that the standard is made up in a medium exactly similar to that which constitutes the unknown solution. This need must also apply to urine, since the latter even after high dilution contains

small amounts of urinary pigments which are considerably enhanced during the course of the diazotization process.

The method presented here overcomes these difficulties by employing a special serum protein-sulphonamide standard for the direct diazotization procedure, and a suitable choice and sequence of reagents.

The modified Marshall method introduced for whole blood is designed on the basis of the principles and conditions already enunciated, which ensures a more quantitative recovery of sulphonamide.

In conclusion, it may be of interest to note that colorimetric assay of the colours given by 693 and sulphanilamide when diazotized and coupled with dimethyl-a-naphthylamine, that weight for weight the intensity is apparently the same, but that the 693 coloration shows a slightly more bluish tint than the sulphanilamide solution. For exact values, therefore, the standard chosen should be the same sulphonamide compound as that administered to the patient.

SUMMARY.

A simple and rapid method is described for the routine determination of sulphonamide in body fluids, the principle being based on the direct diazotization and coupling of dimethyl-a-naphthylamine with sulphonamide in the presence of the proteins of the cerebro-spinal fluid and blood serum. No loss of sulphonamide can therefore occur by adsorption on the precipitated proteins, while an improvement in colour-tint and colorimetric matching results by reason of the excellent buffer effect of the protein itself.

I wish to thank Dr. T. W. Stallybrass, County Medical Officer of Health for Dorset, for permission to use the laboratory for the above investigation, and Dr. T. V. Cooper in granting laboratory facilities and for his encouragement. I am also indebted to Dr. E. J. Gordon Wallace, Medical Officer of Health for Weymouth and M.O. in charge of Weymouth Fever Hospital, for co-operation in supplying the biological material on which this work is based.

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THE EFFECTS OF SODIUM HEXAMETAPHOSPHATE (CALGON) ON THE COMPLEMENT ACTION OF SERUM.

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Received for publication July 30, 1941.

THERE is now a considerable volume of evidence to show that complement action is associated with the protein constituents of serum. That non-dialysable calcium compounds linked to the protein might have a bearing on this problem was suggested by Gordon, Whitehead and Wormall (1926*b*). Citrated plasma shown by Welch, Brewer and Hunter (1940) to possess complement activity, a fact also confirmed by ourselves, would, however, rather militate against the view that calcium is involved in this reaction.

Within the last few years sodium hexametaphosphate has become of increasing technical importance, as this material has the property of removing calcium and magnesium ions from solution and is therefore capable of removing the last traces of hardness in water. This chemical is marketed under the trade name of "Calgon," and a large quantity is now used for scouring and cleaning various textile fabrics. The view is generally held that the calcium, magnesium or indeed other metal ions are converted into complex anions of the type $[M_2(PO_3)_6]^=$, where M is a divalent metal. Another reaction given by hexametaphosphates is the formation of addition compounds with proteins, a well known example being the turbidity produced in acidified albumin solutions after addition of sodium hexametaphosphate solution. In fact it is possible to convert animal skin into a form of white leather by using Calgon in the presence of hydrochloric acid and common salt, the latter being used to check undue swelling of the skin by the acid.

As a consequence of these properties of sodium hexametaphosphate it was determined to investigate the effects of this chemical on the complement action of serum. The material employed was "Calgon T" in the form of glassy plates of about 1/16 inch in thickness, which dissolve easily in water to give a solution of about pH 6·2. Such solutions were invariably adjusted to pH 7·4.

EFFECTS OF SODIUM HEXAMETAPHOSPHATE AND CALCIUM IONS.

The first series of experiments were as follows: Quantities of 0·3, 0·4, 0·5 and 0·6 c.c. of 10 per cent. Calgon in distilled water and adjusted to pH 7·4 were added to 3·0 c.c. quantities of normal guinea-pig serum. The mixtures were then allowed to stand on the bench for 2 hours, after which quantities of 0·1 c.c. of the mixtures were added to 0·5 c.c. of a 4 per cent. suspension of

sensitized ox-cells in 0·85 per cent. sodium chloride. After incubation at 37° C. for two hours the degree of haemolysis was read. In this series of experiments it was noted that only partial haemolysis was obtained when 0·3 c.c. of Calgon solution was employed. With quantities higher than 0·3 c.c. no haemolysis was observed. These experiments show clearly that complement activity is removed by the addition of certain concentrations of Calgon to the serum. This may be due to either :

(*a*) The removal of calcium ions from the serum ; or—

(*b*) The effect of the hexametaphosphate on the serum proteins.

In the second series of experiments, quantities of 0·2, 0·3, 0·4 c.c. of 2·5 per cent. calcium chloride solution adjusted to pH 7·4 were added to 1 c.c. quantities of those mixtures in which the removal of complement activity had been effected by the Calgon treatment after the period of two hours' standing on the bench. A heavy precipitate was noted with the tubes containing 0·3 and 0·4 c.c. of calcium chloride solution and only a slight one with the 0·2 c.c. tube. The tubes were centrifuged to remove the precipitate, and 0·1 c.c. of the clear solution added to 0·5 c.c. of the 4 per cent. suspension of sensitized ox cells. Haemolysis was complete in the cases where heavy precipitates had been noted. Where precipitation had not been complete, as for example where 0·2 c.c. of calcium chloride solution had been employed, partial haemolysis usually occurred. A control series of tubes were set up in which Calgon was added to serum heated to 55° C. for ½ hour to destroy complement and then calcium chloride solution added as described above. The addition of calcium chloride caused similar precipitates, but after centrifuging, 0·1 c.c. quantities tested as before showed no haemolysis, indicating that the calcium chloride itself was not haemolytic.

From these experiments it is clear that the addition of calcium chloride precipitates the excess of hexametaphosphate. The restoration of complement may be due to either :

(*a*) Replacement of calcium ions previously removed by hexametaphosphate, or—

(*b*) The removal of hexametaphosphate combined with the serum proteins.

It will also be noted that the concentrations of calcium chloride employed were not high enough to bring about the removal of complement activity as noted by Wright and MacCallum (1922).

In view of the possibilities outlined above, a third series of experiments was carried out to see if calcium ions were necessary for complement action. To three quantities of 2 c.c. guinea-pig serum were added 2 c.c. normal saline and (*a*) 0·25, (*b*) 0·5, and (*c*) 1·0 c.c. of saturated ammonium oxalate solution. The tubes were allowed to stand on the bench for two hours. Calcium oxalate was precipitated ; the supernatant liquid was poured off and titrated for complement activity. A control guinea-pig serum untreated with ammonium oxalate was diluted in a similar manner and also tested for complement activity. It was found that the complement titre of all these sera was not diminished by the removal of calcium ions. Determinations of calcium on the deposits (kindly carried out by Dr. F. S. Fowweather) resulted as follows : (*a*) 7·2 mg., (*b*) 7·5 mg., (*c*) 7·5 mg. Ca per 100 c.c. serum.

Care was observed in using sodium chloride free from calcium salts in diluting the sera for titration purposes.

This set of experiments makes it quite clear that the removal of calcium ions does not interfere with complement activity.

Thus the restoration of complement activity by addition of calcium chloride noted in the second series of experiments is not due to the replacement of calcium ions, and therefore the alternative explanation that calcium chloride is effective by removing the hexametaphosphate from combination with the serum proteins would appear to be more likely. The question now arises as to whether sodium hexametaphosphate functions as a salt causing dispersion of the serum proteins, lyotropic effect, or as a species of tanning agent, chemical combination with protein, as demonstrated by Wilson (1937).

TABLE I.

No. of tube.	Serum used. (c.c.)	Result.	No. of tube.	Serum used. (c.c.)	Result.
1	0·1 A	3	40	0·1 P + 0·1 F	0
2	0·1 A + 0·1 heated	4	41	0·1 P + 0·1 L	4
3	0·1 A + 0·1 2% egg	4	42	0·1 Q + 0·1 B	3
4	0·1 A + 0·9 saline	4	43	0·1 Q + 0·1 F	0
5	0·1 B	0	44	0·1 Q + 0·1 L	4
6	0·1 B + 0·1 heated	3	45	0·1 R + 0·1 B	2
7	0·1 B + 0·1 2% egg	0	46	0·1 R + 0·1 F	0
8	0·1 B + 0·9 saline	0	47	0·1 R + 0·1 L	3
9	0·1 C	0	48	0·1 S + 0·1 B	2
10	0·1 C + 0·1 heated	0	49	0·1 S + 0·1 F	0
11	0·1 C + 0·1 2% egg	0	50	0·1 S + 0·1 L	3
12	0·1 C + 0·9 saline	0	51	0·1 P + 0·1 C	0
13	0·1 D	0	52	0·1 P + 0·1 G	0
14	0·1 D + 0·1 heated	0	53	0·1 P + 0·1 M	4
15	0·1 D + 0·1 2% egg	0	54	0·1 Q + 0·1 C	0
16	0·1 D + 0·9 saline	0	55	0·1 Q + 0·1 G	0
17	0·1 heated serum	0	56	0·1 Q + 0·1 M	4
18	0·1 2% egg albumin	0	57	0·1 R + 0·1 C	0
19	0·1 P	3	58	0·1 R + 0·1 G	0
20	0·1 P + 0·1 heated	4	59	0·1 R + 0·1 M	3
21	0·1 P + 0·1 A	4	60	0·1 S + 0·1 C	0
22	0·1 P + 0·1 E	0	61	0·1 S + 0·1 G	0
23	0·1 P + 0·1 K	4	62	0·1 S + 0·1 M	2
24	0·1 Q	0	63	0·1 P + 0·1 D	0
25	0·1 Q + 0·1 heated	4	64	0·1 P + 0·1 H	0
26	0·1 Q + 0·1 A	4	65	0·1 P + 0·1 N	4
27	0·1 Q + 0·1 E	0	66	0·1 Q + 0·1 D	0
28	0·1 Q + 0·1 K	4	67	0·1 Q + 0·1 H	0
29	0·1 R	0	68	0·1 Q + 0·1 N	4
30	0·1 R + 0·1 heated	4	69	0·1 R + 0·1 D	0
31	0·1 R + 0·1 A	4	70	0·1 R + 0·1 H	0
32	0·1 R + 0·1 E	0	71	0·1 R + 0·1 N	3
33	0·1 R + 0·1 K	3	72	0·1 S + 0·1 D	0
34	0·1 S	0	73	0·1 S + 0·1 H	0
35	0·1 S + 0·1 heated	4	74	0·1 S + 0·1 N	2
36	0·1 S + 0·1 A	4	75	0·1 K	0
37	0·1 S + 0·1 E	0	76	0·1 L	0
38	0·1 S + 0·1 K	2	77	0·1 M	0
39	0·1 P + 0·1 B	3	78	0·1 N	0

Effect of Dilution.

Gordon and Thompson (1933) have shown that additions of salts have the effect of removing complement activity, and the order of the degree of effectiveness follows the Hofmeister series of ions. Dilution of the various salt solutions, however, led to the restoration of haemolytic complement, showing that the action of the salts was not irreversible.

During the course of this investigation a large number of experiments have been carried out and most of the results have been collected in Table I. In Table I the following sets of tubes demonstrate that dilution with normal saline of sera containing hexametaphosphate in concentrations which are sufficient to inactivate complement does not bring about a restoration : Nos. 5 and 8 ; Nos. 9 and 12 ; Nos. 13 and 16. It will be noted that the original concentration of hexametaphosphate has been reduced to one-tenth without restoration of complement activity. Had the effect of hexametaphosphate been simply of a lyotropic nature such a degree of dilution would have restored the complement activity. The conclusion must therefore be drawn that there is chemical combination of hexametaphosphate with the serum proteins.

There appears to be a similarity between the actions of hexametaphosphate and Congo Red in inactivating complement. Each can have its action reversed, the former by addition of calcium chloride and the latter is removed by adsorption on charcoal (Gordon, 1930). Neither is affected by dilution.

KEY TO TABLE.

A, B, C, D : These represent amounts of 5 per cent. Calgon solution added to 2 c.c. of normal guinea-pig serum :

A, 0·2 c.c. ; B, 0·3 c.c. ; C, 0·4 c.c. ; D, 0·5 c.c.

E, F, G, H : These represent amounts of 5 per cent. Calgon solution added to 2 c.c. of normal guinea-pig serum which had been previously heated to 55° C. for ½ hour and then cooled.

E, 0·2 c.c. ; F, 0·3 c.c. ; G, 0·4 c.c. ; H, 0·5 c.c.

K, L, M, N : These represent amounts of 5 per cent. Calgon solution added to 2 c.c. of normal guinea-pig serum previously heated as described above. Quantities of 1·6 c.c. of these solutions were then treated with 0·6 c.c. of 2½ per cent. calcium chloride solution and centrifuged to remove the deposit.

K, 0·2 c.c. ; L, 0·3 c.c. ; M, 0·4 c.c. ; N, 0·5 c.c.

P, Q, R, S : These represent amounts of N/1 ammonia added to 4 c.c. of normal guinea-pig serum, incubated for 1½ hours at 37° C. and then equivalent amounts of N/1 hydrochloric acid added to bring the reaction to pH values between 7·0 and 7·5.

P, 0·2 c.c. ; Q, 0·3 c.c. ; R, 0·4 c.c. ; S, 0·5 c.c.

" heated " in the table means heated serum as described above.

" 2% egg " means a 2 per cent. solution of egg albumin.

" saline " means normal saline, that is 0·85 per cent. solution of pure sodium chloride.

The numbers in the results column relate to the degree of haemolysis when 0·5 c.c. of a 4 per cent. suspension of sensitized ox-blood cells have been added to the other contents of the tubes and incubated at 37° C. for 2 hours.

4 is complete haemolysis.
3 is nearly complete haemolysis.
2 is partial haemolysis.
1 is trace of haemolysis.
0 is no haemolysis.

It will be noted in Table I that the addition of heated serum restores complement activity to serum previously inactivated by sodium hexametaphosphate (tubes Nos. 5 and 6). This is similar to the effect produced on Congo Red inactivated serum, where it was assumed that on the addition of heated serum a redistribution of the dyestuff occurred whereby the Congo Red was partially removed from the original serum and transferred to the heated serum. The reversal of Congo Red could also be effected by addition of egg albumin solution, but apparently albumin does not absorb hexametaphosphate to any material extent (tubes Nos. 7, 11, 15). There is, however, a slight indication of such adsorption as is shown in tubes Nos. 1 and 3. On the other hand, in tubes Nos. 9 and 10, addition of heated serum does not restore complement activity previously removed by the addition of sodium hexametaphosphate. The reason for this behaviour must be that if the concentration of hexametaphosphate exceeds a certain amount, the excess reacts with the proteins of the heated serum and so inhibits their power of restoring haemolytic complement. In previous work the relation between calcium content and the fourth component of complement (Gordon, Whitehead and Wormall, 1926*a*) had been discussed. It is known that ammonia-treated serum and also hexametaphosphate-treated serum are each devoid of complement activity. In ammonia treated serum it is the fourth component that is inactivated. If the fourth component is not affected by hexametaphosphate, mixing the two inactivated sera should bring about a restoration of complement activity. Actually this is so, as may be seen from consideration of the tubes Nos. 5, 24 and 42. Tube No. 5 represents serum inactivated completely with the minimum amount of hexametaphosphate, tube No. 24 is ammonia inactivated serum, and tube No. 42 is a mixture of the contents of the other two tubes. It will be seen that in tube No. 42 complement activity has been restored. In similar sets of tubes (Nos. 9, 24 and 54), where more hexametaphosphate had been employed there was no restoration of complement, indicating that excess of hexametaphosphate over that required to inactivate serum becomes available to react in the mixture, and the conclusion must be drawn that hexametaphosphate does not inactivate fourth component. The results shown in the table demonstrate clearly the importance of threshold or minimal quantities necessary for inactivation and re-activation. Further examples of the quantitative aspect of these experiments are provided in the series of tubes Nos. 5, 24, 42 and 43, to some of which reference has already been made. Tube No. 5 shows the inactivation of complement by the minimal amount of hexametaphosphate, tube No. 24 shows inactivation by ammonia treatment, also in minimal quantity, tube No. 42 shows the restoration of complement by mixture of sera treated respectively with hexametaphosphate and with ammonia. Tube No. 43 shows that serum first inactivated by heat and then treated with hexametaphosphate is no longer capable of restoring complement activity when added to ammonia inactivated serum. In fact from the consideration of tubes Nos. 19 and 22, there is evidence that hexametaphosphate-heated serum is anti-complementary. A suggested explanation for this behaviour is as follows: After heat treatment the serum proteins are not as reactive to hexametaphosphate as before. Thus there is a larger amount of free hexametaphosphate which is sufficient to react with the serum proteins

incompletely inactivated by the ammonia, and so prevents the restoration of complement activity. The effect of hexametaphosphate on heat-inactivated serum is not irreversible. It is possible to restore complement activity in ammonia-inactivated serum if calcium chloride be added to heated serum treated with hexametaphosphate. An inspection of the table shows this behaviour in an unmistakable fashion. Actually there are four series of tubes that may be examined. These are : (*a*) Tubes Nos. 23, 28, 33 and 38 ; (*b*) tubes Nos. 41, 44, 47 and 50 ; (*c*) tubes Nos. 53, 56, 59 and 62 ; (*d*) tubes Nos. 65, 68, 71 and 74.

Some of the tubes mentioned above include those where initially large additions of hexametaphosphate have been made. Normally these would have completely removed complement activity, but the addition of calcium chloride has removed the hexametaphosphate from combination with the protein.

EFFECT OF PHOSPHORIC ACIDS.

It was considered desirable to test the effects of the salts of the different phosphoric acids on the inactivation of complement activity. It is well known that only metaphosphates precipitate albumin from acid solution, and they are also the only phosphoric acid derivatives with tanning properties on animal skins. Di-sodium orthophosphate was found to be ineffective in its action on serum complement, but sodium pyrophosphate had a slight action in removing complement, but only in relatively high concentration and was reversible on dilution. It can therefore be fairly concluded that pyrophosphate acts through its comparatively high dispersing effect on the protein aggregates, and in fact it has been used in the leather industry for facilitatiug the soaking of hard dried skins to a state of softness and suppleness usually encountered with ordinary fresh hides and skins. The softening effect is ascribed to the solution of dried inter-fibrillary proteins which are akin to hard-boiled white of egg.

The following is a brief summary of the results obtained using 5 per cent. solutions of sodium salts of the various phosphoric acids which had been adjusted to pH 7·4. Varying amounts of the different phosphate solutions were added to 1 c.c. of normal guinea-pig serum :

c.c. of 5 per cent. solution.	Type of phosphate.	Complement inactivation.
0·1	Calgon	None.
0·2	,,	Complete.
0·8	Sodium pyrophosphate	Partial.
0·9	,, ,,	Complete.*
1·0	,, orthophosphate	None.

* On dilution with saline complement activity was restored.

ACTION OF HEXAMETAPHOSPHATE ON THE IMMUNE BODY.

There remains the somewhat remote possibility that the inactivation of complement is not due to the reaction between the serum proteins and hexa-

metaphosphate, but is the result of the action of the latter on the immune body absorbed on the red blood cells. In consequence of this possibility quantities of Calgon in amounts previously found to be in excess of that required for inactivation of complement were added to guinea-pig serum. This treated serum produced no haemolytic action on sensitized red blood cells. The latter were then spun down on the centrifuge, supernatant liquid removed and the cells further washed by successive centrifuging operations. Addition of untreated guinea-pig serum now gave complete haemolysis. It is thus clear that hexametaphosphate has no permanent effect on sensitized red blood cells in preventing haemolysis as it has on the proteins of serum.

The tanning action of hexametaphosphates is obviously due to the reaction between the basic groups of the protein and the complex anion of the hexametaphosphate. In order that this reaction should proceed it is necessary for acid to be present, and this has the effect of back-titrating the negatively charged $-COO^-$ parts of the protein "Zwitterions" and converting them into uncharged $-COOH$ groups. The positively charged $-NH_3$ groups of the protein side chains are then free to combine with the hexametaphosphate anions to form the insoluble precipitates with protein solutions, such as those of albumin or the analogous leather when the protein is in a fibrous condition as is the case with hide. In the experiments described in this paper the reactions have taken place at pH 7·4 and the protein "Zwitterions" are intact. Nevertheless a reaction does take place to a certain extent in which the complex anion of the hexametaphosphate must be competing with the protein anions for combination with the positively charged basic groups of the proteins in serum. Apparently this reaction need not be very far-reaching in order to remove the complement activity.

SUMMARY.

Sodium hexametaphosphate (Calgon) when added to serum removes complement activity.

This effect is reversed by the addition of calcium chloride.

Hexametaphosphates remove calcium ions from solution, but the restoration of complement activity is not to be ascribed to the replacement of calcium, but is to be explained by calcium chloride removing hexametaphosphate from combination with the serum proteins.

The inactivation by Calgon is not an ordinary lyotropic salt effect, because dilution does not restore complement activity.

Hexametaphosphates and Congo Red exhibit parallel effects both in the inactivation of complement and in reversibility.

Hexametaphosphates behave differently in their reactions with proteins from salts of other varieties of phosphoric acid. This applies to the action on serum complement and on the tanning of hides and skins.

We should like to express our indebtedness to the Medical Research Council for a grant towards the expenses of this work.

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STUDIES ON THE EFFECT OF CARCINOGENIC HYDROCARBONS ON IMMUNITY REACTIONS.

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Received for publication August 7th, 1941.

IN the present experiments the effect of carcinogenic hydrocarbons on the formation and the amount of precipitating antibodies has been investigated.

The experiments are divided into two main lines :

(1) The effect of treatment with carcinogenic hydrocarbons, prior to immunization, on the formation of antibodies.

(2) The effect of carcinogenic agents, applied at the stage of high immunity, on the degree of immunity.

For the estimation of the amount of antibodies generally two methods have been used : the method of optimal proportions of Dean and Webb (1926), and the determination of the amount of nitrogen in the antigen-antibody precipitate. In some of the experiments the antibodies were estimated by the dilution method.

PART I.

Four rabbits received polycyclic hydrocarbons during one year. One animal was treated with methylcholanthrene, one with 1 : 2 : 5 : 6-dibenzanthracene, one with 1 : 2-benzanthracene and one with phenanthrene. The hydrocarbon was injected as a 0·05 per cent. colloid in a 1 per cent. watery gelatine solution. The injections were given subcutaneously, 5 c.c. each time at four to eight weeks' intervals. The animals looked healthy at the beginning of the immunization. As antigen, horse serum was injected intravenously twice weekly during two weeks and once intraperitoneally in the third week, 2 c.c. each time. At the end of the second and third week, and 10 days after

the last injection, blood was drawn from the ear vein and the amount of antibody estimated by determining the weakest dilution of antibody with a constant amount of antigen, and also the weakest dilution of antigen with a constant amount of antibody, to give a visible precipitate in one hour at room temperature. All animals developed a strong immunity; there was no difference between the amounts of precipitin produced in the blood of the animals treated with carcinogenic and non-carcinogenic hydrocarbons. Six months later the animal treated with 1 : 2 : 5 : 6-dibenzanthracene underwent a second series of horse serum injections and this produced strong antisera as estimated by the method of Dean and Webb. Fig. 1 shows that the course of the reaction is similar to that in untreated animals.

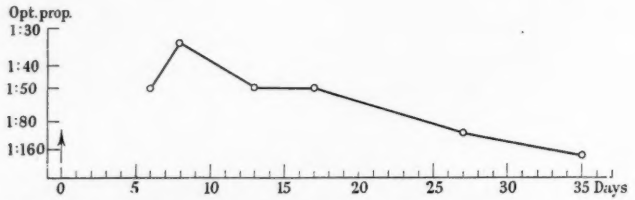

FIG. 1.—Production of antihorse serum precipitins in a rabbit treated with 1 : 2 :5 : 6-dibenzanthracene for 1½ years prior to immunization.

↑ = Last injection of horse serum.

PART II.

Twelve young rabbits of either sex were immunized by injections of 2 c.c. horse serum intravenously twice weekly during three weeks. On the 7th, or 7th and 9th days after the last serum injection, the state of immunity was estimated and 5 c.c. of the colloidal hydrocarbon solution was injected subcutaneously immediately afterwards. The course of immunity was followed by the estimation of immune bodies 48 hours, 3 days, 7 days and about 3 weeks later. The hydrocarbons used were methylcholanthrene, 1 : 2-benzanthracene and anthracene. Table I and Fig. 2 summarize the results obtained by the method of optimal proportions. The precipitations were carried out in a set of 10 to 15 small test tubes (1 c.c. capacity). Progressively increasing volumes from 0·01 c.c. to 0·1 c.c. of antigen diluted 1 : 25 or 1 : 50 were put into separate tubes. The volume was brought up to 0·1 c.c. in all tubes by addition of normal saline, 0·1 c.c. of the diluted antibody (1 : 5) was added and the course of the reactions was followed at room temperature. The amount of the antibody was usually the same in the two samples taken before the hydrocarbon was injected and precipitation occurred after the same time. Forty-eight hours after the injection of 1 : 2-benzanthracene or anthracene the optimal proportion was unchanged or slightly lowered. Of the 9 animals injected with methylcholanthrene, in 7 cases a sudden drop occurred within 48 hours and the time of precipitation was generally prolonged. In one animal (314) the amount of antibodies declined also before the injection of the carcinogenic hydrocarbon, and in another rabbit (302) no change occurred

TABLE I.—*Optimal Proportions and Time of Precipitation.*

No. of rabbit	Hydrocarbon injected.	Days after last injection of horse serum.		Days after injection of hydrocarbon. −1 to −3.	0.	1.	2.	4.	7.	13.	21.	23.
328	Methyl-cholanthrene	8	Ratio	1 : 10	1 : 10	..	1 : 50	..	1 : 66·7	..	No immunity	
			Time	17	17	..	25	..	23			
359	,,	8	Ratio	1 : 12·5	..	..	1 : 25	1 : 50	No immunity			
			Time	15	..	..	20	..	—			
378	,,	6	Ratio	..	1 : 11	..	1 : 25	..	1 : 50	No immunity		
			Time	..	13	..	18	..	28			
324*	,,	10	Ratio	1 : 6	1 : 6	1 : 9·6	1 : 12					
			Time	12	12	12	25					
316	,,	9	Ratio	1 : 14·3	1 : 14·3	..	1 : 33	..	1 : 33	..	No immunity	
			Time	15	13	..	50	..	75	..		
314	,,	9	Ratio	1 : 10	1 : 14·3	..	1 : 16	..	1 : 32	No immunity		
			Time	18	20	..	80	..	60			
302*	,,	8	Ratio	1 : 16	1 : 16	1 : 16						
			Time	30	30	90						
325	1 : 2-benz-anthracene	10	Ratio	1 : 9·6	1 : 9·6	1 : 12	1 : 12					
			Time	20	25	25	27					
330	Anthracene	8	Ratio	1 : 16·7	..	..	1 : 16·7	..	1 : 25	..	..	1 : 100
			Time	15	..	..	15	..	20			
329	,,	8	Ratio	1 : 16·7	..	..	1 : 25	..	1 : 33	..	..	Traces
			Time	28	..	..	35	..	43			

Antibody diluted 1 : 10. Ratio : Antigen-antibody ratio at the point of most rapid flocculation.
Antigen diluted 1 : 50. Time : Time of flocculation in minutes.
Total amount of reacting fluid 0·2 c.c. * 0·2 of rabbit serum.

after the injection of the hydrocarbon apart from a prolongation of the time of precipitation from 30 to 90 minutes. No antibody could be detected in the sera of animals treated with methylcholanthrene after 15–29 days after the last injection of horse serum, while in the animals which received anthracene a definite immunity could still be demonstrated on the 31st day. Two animals on which the dilution method was used are not included in the table. In principle they seemed to react in a similar manner.

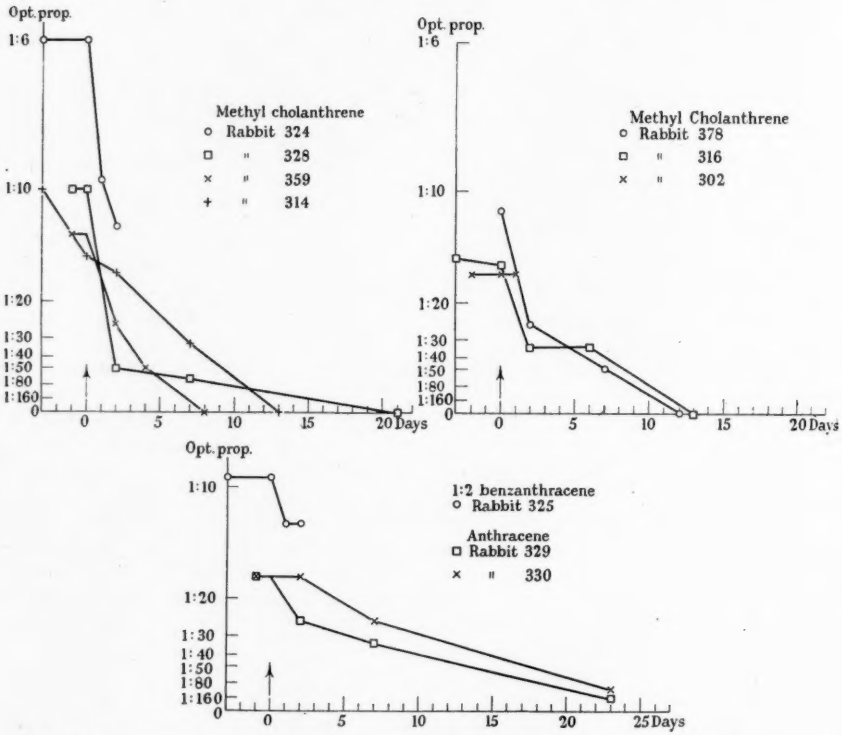

FIG. 2.—Changes in the optimal proportions of the sera of immunized rabbits after injection of hydrocarbon.

↑ = Injection of 5 c.c. of a 0·05 per cent. solution of hydrocarbon.

The amount of nitrogen was estimated in the precipitate formed by the addition of three different proportions of antigen to a constant volume of antiserum. The mixture of serum and antigen was allowed to stay in the ice-chest overnight (15 hours). The precipitate was washed three times with saline solution, and the nitrogen determined by a micro-Kjeldahl method (Hoch, 1940). Table II shows that the *N* content in the antibody-antigen precipitate decreases slowly in the days following the injection of anthracene.

Methylcholanthrene induced a strong drop in the amount of nitrogen after 48 hours. The decrease in the nitrogen after injection of anthracene was 4 to 18 per cent., and 17 to 33 per cent. after injection of methylcholanthrene. The decrease in the amount of nitrogen in the precipitate from animals injected with methylcholanthrene, although greater than that from animals injected with anthracene, is not proportional to the decrease of antibody shown by the method of optimal proportions.

DISCUSSION.

In the present experiment a difference was found between the effect of carcinogenic hydrocarbons applied through a long period prior to immunization, and of treatment with these hydrocarbons at the peak of immunity. While the prolonged treatment previous to immunization did not seem to influence the ability to produce antibodies, an injection at the peak of the immunity decreases markedly the amount of antibodies. In all the immune sera the amount of antibodies gradually decreases about 10 days after the last injection of antigen. The progressive decline after injection of anthracene, applied on the 8th day after the last injection of horse serum, may be due to this, but the sudden drop within 48 hours after the injection of methylcholanthrene must be attributed to a special effect of this hydrocarbon.

The formation of antibodies is generally supposed to be localized in the cells of the reticulo-endothelial system. Deutsch showed in 1899 that splenectomy previous to immunization to typhoid does not influence the production of antibodies, but splenectomy in the immune animals decreases the grade of immunity. Several recent investigations point to a possible influence of carcinogenic hydrocarbons on the reticulo-endothelial system. Animals treated with carcinogenic hydrocarbons showed generally a decrease of the splenic weight and changes in the histological structure of the spleen (Parsons, 1938; Hoch-Ligeti, 1941). Changes in the rate of elimination of electronegative colloid (Congo-red) from the circulation after injection of carcinogenic hydrocarbons have been demonstrated (Csato, Wetzler-Ligeti and Wiesner, 1939; Hoch-Ligeti, 1940). The parallelism between the response of animals to splenectomy and to the injection of carcinogenic hydrocarbon seems to be a further indirect evidence of a possible connection between the reticulo-endothelium system and an effect of carcinogenic hydrocarbons. Whether and how far this and the neoplastic effect are connected will be the object of further investigations. It is questionable whether an effect manifesting itself within 48 hours, such as the effect on the amount of antibody in the serum, and the production of tumours, which in rabbits takes one or more years, can be directly related, the more so as the prolonged previous treatment has no effect on the production of immune bodies.

In these experiments the amount of the antibodies was estimated by two different methods. The actual figures obtained are not in quantitative agreement. The changes provoked by injection of methylcholanthrene measured by the method of the optimal proportion are much greater than when measured by the estimation of the amount of nitrogen in the precipitate. This

TABLE II.—*Amount of Nitrogen in Antibody-Antigen Precipitates.*

A. *Methylcholanthrene.*

0·4 c.c. of rabbit serum diluted 1 in 5 was added to each dilution of horse serum.

Rabbit No. 328.

Days after last injection of horse serum	8	10	15	29
Days after injection of hydrocarbon	0	2	7	21

c.c. of horse serum diluted to 1/25.	Proportion AG/AB.	Amount of nitrogen in μg.				Drop in percentage within 48 hours after injection of hydrocarbon.
0·035	1/57	..	..	67	No immunity	..
0·1	1/20	124	85	..	..	32
0·2	1/10	117	84	..	..	28
0·3	1/6·7	106	80·5	..	..	24

Rabbit No. 359.

Days after last injection of horse serum	7	10	12	16
Days after injection of hydrocarbon	−1	2	4	8

c.c. of horse serum diluted to 1/25.	Proportion AG/AB.	Amount of nitrogen in μg.				Drop in percentage within 48 hours after injection of hydrocarbon.
0·02	1/100	..	..	25·5	No immunity	..
0·04	1/50	46	38	36·5	..	17
0·08	1/25	66	53	47·5	..	20
0·16	1/12·5	72	56·5	..	..	21

Rabbit No. 378.

Days after last injection of horse serum	6	8	..	18
Days after injection of hydrocarbon	0	2	..	12

c.c. of horse serum diluted to 1/50.	Proportion AG/AB.	Amount of nitrogen in μg.				Drop in percentage within 48 hours after injection of hydrocarbon.
0·08	1/50	57·5	47·5	No immunity		17
0·16	1/25	73	49	..	..	33
0·36	1/11	78	54·5	..	..	30

B. *Anthracene.*

0·4 c.c. of rabbit serum diluted 1 in 5 was added to each dilution of horse serum.

Rabbit No. 330.

Days after last injection of horse serum	7	10	15	..
Days after injection of hydrocarbon	−1	2	7	..

c.c. of horse serum diluted	Proportion AG/AB.	Amount of nitrogen in μg.				Drop in percentage within 48 hours after injection of hydrocarbon.
0·04	1/50	..	..	61·5.	..	..
0·05	1/40	88	84	..	..	5
8	1/25	..	..	73	..	..
2	1/16·7	133·5.	127·5.	92	..	4
5	1/8	159	..	..	..	..

Rabbit No. 329.

Days after last injection of horse serum	7	10	15	..
Days after injection of hydrocarbon	−1	2	7	..

c.c. of horse serum diluted to 1/25.	Proportion AG/AB.	Amount of nitrogen in μg.				Drop in percentage within 48 hours after injection of hydrocarbon.
0·04	1/50	..	..	40·5.	..	..
0·05	1/40	69	62	..	..	10
0·08	1/25	..	..	43·5.	..	..
0·12	1/16·7	72·5.	61·5.	45	..	15
0·25	1/8	70	57·5.	..	..	18

AG = Antigen.
AB = Antibody.

discrepancy may be due to the difference in the time when the reactions were read. The readings of the optimal proportions were taken at the moment of precipitation. For the estimation of nitrogen, the precipitation was allowed to stand 15 hours in the ice-box. American authors assume (Heidelberger, Treffers and Mayer, 1940 ; Pappenheimer, 1940) that the immune bodies may contain a " monovalent " or incompletely reacting part, which is soluble, and a " multivalent " precipitable part. The " monovalent " part may be rendered precipitable by the presence of antibody-antigen precipitate. One could suggest that by the injection of methylcholanthrene one part of the antibody became " monovalent " and as such did not appear when the method of the optimal proportion was used. After standing overnight it might have joined the formed precipitate and increased the nitrogen estimated.

SUMMARY.

Prolonged treatment with carcinogenic hydrocarbons previous to immunization did not influence the ability of rabbits to produce precipitating antibodies.

In immune rabbits the injection of a carcinogenic hydrocarbon provokes a sudden drop in the amount of the immune bodies within 48 hours.

I am much indebted to Prof. J. R. Marrack for help and advice throughout the course of this investigation. It is a pleasure to express my thanks to the Sir Halley Stewart Trust for a fellowship held during this work. The British Empire Cancer Campaign makes a grant in support of the Institute where this work was carried out.

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